PATHWAYS IN BIOSYNTHESIS OF PLANT GALACTOLIPIDS

AERNOUT VAN BESOUW

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PROMOTOR: DR.IR. J.F.G.M. WINTERMANS

COPROMOTOR: PROF.DR. H.F. LINSKENS

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Ter nagedachtenis aan mijn vader

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CONTENTS

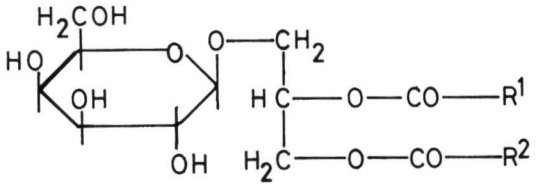
ACP	acyl carrier protein
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
buffer 2	0.1 M tricine/KOH pH 7.2, 10 mM $MgCl_2$
CDP	cytidine diphosphate
CMP	cytidine monophosphate
CoA	coenzyme A
DGDG	digalactosyl-diacylglycerol (digalactolipid)
dpm	desintegrations per minute
IEF	iso electrical focussing
MGDG	monogalactosyl-diacylglycerol
NAD(P)H	the reduced form of nicotinamide adenine dinucleotide (phosphate)
NMR	nuclear magnetic resonance
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PI	phosphatidyl inositol
SQDG	sulphonoquinovosyl-diacylglycerol (sulpholipid SL)
TeGDG	tetragalactosyl-diacylglycerol
T(e)GDG	trigalactosyl-diacylglycerol + tetragalactosyl-diacyl-glycerol
TGDG	trigalactosyl-diacylglycerol
Tricine	N{2hydroxy-1,1-bis(hydroxymethyl)ethyl}glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine diphosphate
UDPG	uridine diphosphate glucose
UDPGal	uridine diphosphate galactose
UMP	uridine monophosphate
UTP	uridine triphosphate

1 INTRODUCTION

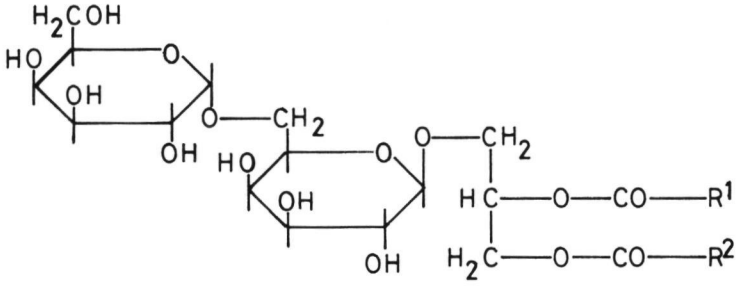
1.1 GENERAL INTRODUCTION

For many years chloroplast lipids have been a subject of research in our laboratory (Wintermans and De Mots 1965). During this span more attention has been focussed on galactolipids (Helmsing 1967, 1970a, b, Wintermans *et al.* 1969, Wintermans 1971) because they were assumed to be involved in one way or another in the photosynthetic mechanism, since they represent the main lipids of the chloroplast (Wintermans 1960, Bailey and Whyborn 1963). In fig. 1.1 the structural formulas of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are shown. The galactose is linked to the glycerol with a β-glycosidic bond, whereas the bond between 2 galactosyl moieties is always α-glycosidic (Carter *et al.* 1956, 1961). Over 50% of the lipids in chloroplasts are mono- and digalactosyldiacylglycerol, although both trigalactolipid (TGDG) (Galliard 1969) and tetragalactolipid (TeGDG) (Joyard and Douce 1976b) have been demonstrated to occur in chloroplast extracts. A rather different glycolipid is sulphoquinovosyldiacylglycerol (SQDG) (fig. 1.1) The sulpholipid differs from the other glycolipids because of the strongly negatively charged sulphonic acid group. Moreover it has also a specific fatty acid composition differing markedly from MGDG and DGDG and possibly indicating a different function in the chloroplast membranes (Heinz 1977).

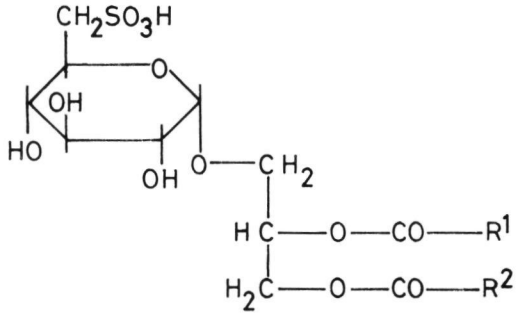
Fig. 1.1



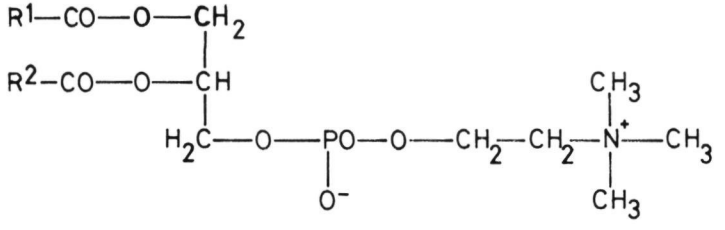
3-O-β-D-galactopyranosyl-*sn*-1,2-diacylglycerol (monogalactolipid or MGDG)



3-O-{-α-D-galactopyranosyl-(1'→6')-O-β-D-galactopyranosyl}-*sn*-1,2-diacyl-glycerol (digalactolipid or DGDG)



6-sulfo-α-D-quinovopyranosyl-(1→3')-1',2'-diacyl-*sn*-glycerol (sulfolipid or SQDG)



3-*sn*-phosphatidyl-choline (lecithin or PC)

Flowers (Thompson *et al.* 1968, Liedvogel and Kleinig 1976), storage organs (Galliard 1968a, Lepage 1968), fruits (Galliard 1968b) and seeds (Carter 1956) are known to contain galactolipids, but they are mostly found in the leaves.
Within the plant cell, galactolipids are localised mainly in plastids, *i.e.* in chloroplasts (Wintermans 1960), in etioplasts (Hawke *et al.* 1974), in chromoplasts (Liedvogel and Kleinig 1976) and in amyloplasts (Lepage 1968). Considerable differences in fatty acid composition may be found depending on species, organelles and stage of development (Bahl *et al.* 1976, Duval *et al.* 1976). At present there is a tendency to confine the presence of galactolipids to plastid membranes. The observation of Ongun and Mudd (1968) that only 85% of the galactolipids could be assigned to chloroplasts, as well as the presence of galactolipids in the plasmalemma (Lamant and Heller 1975), in mitochondria (Schwertener and Biale 1973) and in micrsomes (Oursel *et al.* 1973) can be ascribed to imperfect isolation procedures. One of the most serious objections to this hypothesis is the observation of Mackender and Leech (1974) that in mitochondria of *Vicia faba* galactolipids occur with a fatty acid composition completely different from the galactolipids in chloroplasts thus excluding the possibility of cross contamination.
Douce *et al.* (1973) observed in spinach chloroplasts a ratio of MGDG/DGDG equal to 2. Hashimoto and Murakami (1975) reported ratios of 2 and 1.7 for this species. In *Vicia faba* it was found to be 2.5 (Mackender and Leech 1974) and in *Beta vulgaris* 1.8 (Nichols and James 1968), all on the basis of weight.
In 1973 Douce *et al.*, and Poincelot succeeded in fractionating chloroplasts into 3 subfractions, being the photosynthetic lamellae, the stroma and the envelope. The latter of these fractions separates the chloroplast stroma from the cytoplasm.

Since 98% of the total lipid of chloroplasts can be found in the lamellae, it is not surprising that the MGDG/DGDG ratios in lamellae equal those of intact chloroplasts. In marked contrast the envelopes have a very different lipid composition with reported MGDG/DGDG ratios of 0.3-1 (table 6.1). Also the content of linolenic acid, which is believed to be essential in plant tissues for the formation of grana (Blee and Schanz 1978) is much lower in envelopes than in lamellae. In spinach, Douce *et al.* (1973) reported linolenic acid to amount up to 55% in envelopes but to 70% in lamellae and in *Vicia faba*, Mackender and Leech (1974) calculated that linolenic acid constituted 60% of the total fatty acid in envelopes but 90% in lamellae. The sulpholipid is believed to occur in photosynthetic tissues exclusively in chloroplasts (Davies *et al.* 1965, Ongun *et al.* 1968), where it forms about 5% of the total lipid (Douce *et al.* 1973). In chloroplast envelopes there are contradictory data, ranging from traces of sulpholipid (Poincelot 1976b) to amounts equal to those in lamellae (Douce *et al.* 1973).

1.3 FUNCTION OF GALACTOLIPIDS

Since galactolipids constitute ±80% by weight of the nonpigmented lipids in chloroplasts, several functions have been ascribed to these lipids in the photosynthetic machinery, such as storage of carbohydrate (Benson *et al.* 1959), transport of sugar (Benson 1963) and protection against frost injury (Bervaes *et al.* 1972). Chary and Lundin (1965) even proposed that galactolipids can take part in the light reaction in which the short wavelength system is involved. Of these functions only the protection against frost injury may find some acceptance at present. Although the resistance to low temperature is mainly

caused by a change in fatty acid composition, the MGDG/DGDG ratio also changes (Kuiper 1970). That galactolipids are not directly involved in the photosynthetic mechanism was shown by Shaw *et al.* (1976) by the action of a lipase isolated from runner beans on photosynthetic membranes. To prevent disruption of the membranes by liberated fatty acids or other products of hydrolysis of membrane lipids, the experiments were performed in the presence of albumin. After exhaustive enzymatic hydrolysis, 70% of the MGDG and 50% of the DGDG and lecithin (for structural formulae see fig. 1.1) were removed, but none of the sulpholipid and phosphatidylglycerol. However, the electron flow through photosystems I and II, as measured with artificial electron donors and acceptors, was not affected. Also electron microscopic pictures of the membranes indicated that they were stacked normally. It is of interest that none of the sulpholipid was degraded by the lipase from bean leaves in these experiments, although the same enzyme showed activity towards sulphoquinovosyldiacylglycerol in experiments by Burns *et al.* (1977). At present most of the phospho- and glycolipids are thought to be arranged in lipid bilayers (Singer and Nicholson 1972), in which individual molecules can shift laterally, within the plane of the membrane at fast rates (Devaux and McConnel 1973). Flip-flop movements from one monolayer of the membrane to the other proceed normally at low rates (Kornberg and McConnel 1971), although irregularities in the membrane monolayers, such as proteins or "frozen" lipid domains, may increase the speed of the transbilayer movement at their borders up to several orders of magnitude (van Zoelen 1978). The rate of transbilayer movements also depends on fatty acid composition (de Kruijff and Wirtz 1976) and on the molecular species. For example diacylglycerol is shown to have a much faster rate of flip-flop-movement than phospholipids (Allen *et al.* 1978). Not all lipids in a biomembrane have the same freedom of movement. This freedom may be restricted by several conditions. Proteins, embedded in a biomembrane are thought to have a lipid annulus (Mazliak 1977), a core of lipid

molecules with a reduced mobility, as has been shown by NMR (van Zoelen 1978). These lipids may be attacked to a lesser extent, or even not at all, by lipid degrading enzymes (Bevers 1978). Also the lipids can be hidden from the lipid degrading enzyme by an intact mono- or bilayer. The resistance of sulpholipid to enzymatic hydrolysis may be due to inaccessibility of the lipid to the enzyme.
MGDG and DGDG are probably main elements of the lipid matrix of the chloroplast membranes, although many proteins may contain them in their lipid annulus. The fatty acyl moieties of galactolipids have high rates of turnover (Williams *et al.* 1975a,b, Heinz 1977). This may be due to the need of continuous adjustment of their composition. Linolenic acid, the main constituent fatty acid of photosynthetic tissues in plants, is generally quite liable to autoxidation and the O_2 concentration is very high in illuminated chloroplasts. Although natural antioxidants are present in the chloroplast membrane (e.g. quinones, tocopherols), oxidative degradation of polyunsaturated fatty acids in the light must be considered probably. It is of interest in this connection that the sulpholipid not only has a relatively low content of linolenic acid (45% in spinach chloroplasts, in contrast to 70% for a total chloroplast extract, Douce *et al.* 1973) but that the turnover of sulpholipid fatty acyl moieties is also relatively slow (Heinz and Harwood 1977).

1.4 BIOSYNTHESIS OF GALACTOLIPIDS

1.4.1. *Introduction*

In the biosynthesis of galactolipids several stages can be discerned, which will be discussed separately.

Galactolipids are formed from three precursors (Heinz 1977) which are:

a) glycerol, in the form of *sn*-glycerol-3-phosphate
b) fatty acids, in the form of their CoA thioesters
c) galactose, in the form of UDPGal.

The activated fatty acids are linked to *sn*-glycerol-3-phosphate, forming phosphatidic acid, which is then hydrolysed to 1.2 diacyl-*sn*-glycerol. The latter compound subsequently accepts a galactosylmoiety from UDPGal.

1.4.2 sn-*Glycerol-3-phosphate*

All enzymes required for at least one pathway in the biosynthesis of glycerol-3-phosphate have been demonstrated to be present in leaf homogenates. In this pathway glyceraldehyde-3-phosphate is dephosphorylated to glyceraldehyde (Randall and Tolbert 1971), which in turn is reduced to glycerol in a NADPH dependent reaction (Königs and Heinz 1974). Subsequently the glycerol is phosphorylated to glycerol-3-P by glycerolkinase (Hippmann and Heinz 1978). Glycerol-3-phosphate is directly accessible for acylation within the cytoplasm. It can also be transported across the envelope membrane by the phosphate translocator (Heldt and Rapley 1970) for acylation within the chloroplast.

1.4.3 *Acyl-CoA*

Chloroplasts differ from other plastids in being autonomous for their supply of energy and reducing power: ATP and NADPH. In chloroplasts, acetate can be activated to acetyl-CoA by acetate thiokinase (Kannangara *et al.* 1973). The acetyl-CoA may then be carboxylated to malonyl-CoA (Kannangara and Stumpf 1972). Further chain elongation results in acyl-ACP thioesters (Shine

et al. 1976b). However, the established acyldonors in galactolipids biosynthesis are acyl-CoA thioesters (Heinz 1977). These result from the action of thiokinase on the fatty acids produced by hydrolysis of the acyl-ACP thioesters (Shine *et al.* 1976a,b). A reason for this energy consuming extra reaction step is not yet known.

1.4.4 *Formation of diacylglycerol*

Acylation of *sn*-glycerol-3-phosphate may occur in several compartments. For the biosynthesis of galactolipids only microsomal and plastidic activities seem to be involved (Heinz 1978). The microsomal system from leaves, supplied with glycerol-3-phosphate, incorporates myristic, palmitic, stearic and oleic acyl-CoA thioesters into (lyso)phosphatidic acid and the corresponding dephosphorylated compounds, mono- and diacylglycerol (Sastry and Kates 1966). Phospholipids synthesised in microsomes can be transported by phospholipid exchange proteins (Kader 1975). When the experiments with microsomes were performed with labelled fatty acids instead of labelled glycerol, the reaction appeared to be independent of the amount of glycerol present and the label was incorporated with high specificity into lecithin at the *sn*-2 position (Vijay and Stumpf 1971).
In chloroplasts *sn*-glycerol-3-phosphate is acylated by a soluble enzyme in the stroma to monoacylglycerol-3-P (= lysophosphatidic acid)(Joyard and Douce 1977). In the same series of experiments Joyard and Douce (1977) showed that isolated envelopes catalysed the acylation of lysophosphatidic acid to phosphatidic acid in the presence of ATP and coenzyme A. Phosphatidic acid was degraded to diacylglycerol by the action of a phospholipase C, which was also present in the chloroplast envelope (Joyard and Douce 1977). The diacylglycerol thus generated however, contained predominantly C_{16-0} and C_{18-1} fatty acids, whereas diacylglycerol

in isolated envelopes contains mostly C_{16-3} and C_{18-3} fatty acids (Joyard and Douce 1976a).

1.4.5 *UDPGalactose*

In view of the enzymes present in plant cell homogenates, various pathways are possible in the biosynthesis of UDPGalactose. The pathway with highest enzymic activities considered to be the most plausible, we suggest that UDPGal results from UDPG by the action of UDPG epimerase, localised in the cytoplasm (Heinz 1977). The UDPG in turn is built from UTP and glucose-1-P, this reaction being catalysed by a pyrophosphorylase, which is also present in the cytoplasm (Königs and Heinz 1974, Bertrams and Heinz 1976). Plastids, being specifically concerned with starch metabolism, are equipped with enzymes required for its synthesis and degradation. The nucleotide sugar involved in starch biosynthesis seems to be ADPG rather than UDPG (Murata *et al.* 1964, Lehninger 1975).

1.4.6 *Formation of galactolipids*

Since UDPGal is formed in the cytoplasm and is unable to penetrate through the envelope in the stroma of the chloroplast (Heber 1974) it becomes understandable that the envelope is the site of synthesis of the galactolipids (Douce 1974, van Hummel *et al.* 1975).
A model for the galactolipid forming reaction was first proposed by Benson *et al.* in 1958:

UDPGal + diacylglycerol → MGDG + DGDG I

Similarly, for the formation of DGDG these authors proposed:

UDPGal + MGDG → DGDG + UDP II

Galactose labelled MGDG and DGDG were obtained from incubations of several leaf and chloroplast preparations, supplied with UDPGal (Neufield and Hall 1964, Chang and Kulkarni 1970). Different enzymes were assumed to catalyse reactions I and II, based on observed differences in solubility (Mudd *et al.* 1969), on the results of $^{14}CO_2$ labelling experiments in vivo (Williams *et al.* 1975b) and based on observed differences in pH optima (Joyard and Douce 1976b).

1.5 THE AIMS OF THE PRESENT STUDY

In the experiments reported below we tried to answer 2 questions.

1. Is it possible to establish more closely the site of galactolipid formation within the chloroplast envelope, which consists of two distinct unit-membranes?

This question proved rather untractable. A few pertinent observations will be presented and discussed in chapter 3.

2. Since the incorporation is so complex, is it possible to discern the enzymic activities involved in MGDG and DGDG biosynthesis and to get an insight in factors that regulate the proportions in which both major thylakoid lipids are synthesised?

We systematically studied the effects of differences in incubation time, temperature and pH. Also various effectors were tested, which ultimately led to the results described in chapter 4 and 5. The significance of these results for the composition of envelopes is discussed in chapter 6.

2 MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. *Spinach*

Spinach (*Spinacia oleracea L.*) was bought on the local market or grown in a greenhouse under definite conditions. These were: 25 000 lux during 8 hours at 17°C followed by 16 hours darkness at 13°C. The relative humidity was always over 75%. Leaves were harvested from 5 till 10 weeks after sowing. Spinach from the local market was used immediately, but spinach leaves from the greenhouse were fasted 24 or 48 hours at 4°C in the dark, in order to remove excess of starch, before chloroplasts were isolated.

2.1.2 *Radioactive compounds*

Radioactively labelled UDPGal and UDPG were obtained from the Radiochemical Centre, Amersham; UDP(^{3}H)Gal: batch 3, 4 and 6 specific activity $6.0 \cdot 10^{14}$ Bq/mol; UDP(^{14}C)Gal: batch 23, 27 and 30, specific activity $1.2\text{-}1.3 \cdot 10^{13}$ Bq/mol; UDP(^{14}C)G: batch 34, specific activity $1.1 \cdot 10^{13}$ Bq/mol.
(^{14}C)Dioleoylphosphatidylcholine was a gift from dr. E.J. van Zoelen, Laboratory of Biochemistry, State University, Utrecht.

2.1.3 *Chemicals*

UDPGal, UDPG and ATP, sigma grade, were obtained from the Sigma Chemical Company. ATP, ADP, AMP, UTP, UDP, UMP, CDP, CMP, uridine, uracil, galactose-1-phosphate, glucose-1-phosphate, NADH, NADPH, cytochrome c and phospholipase C (from *Bacillus cereus*) were purchased from Boehringer, Mannheim. Other reagents were, analytical grade, from Merck, Darmstadt.

2.1.4 *Preparation of labelled substances*

By incubation of di(^{14}C)oleoylphosphatidylcholine with phospholipase C (E.C. 3.1.4.3 from *Bacillus Cereus*) we obtained radioactively labelled diolein. Because the diacylglycerol was not easily dissolved properly in the envelope membranes, the lecithin was sonified with an envelope preparation and then the phospholipase was added. Contrary to the statement of the manufacturer, the addition of calcium ions was not necessary. In the presence of chloroplast envelopes and only Mg^{2+} as a divalent cation complete hydrolysis of lecithin was ascertained by thin layer chromatography.
Mono(^{14}C)galactosyldiacylglycerol was obtained by incubation of envelopes at pH 8.0 in the presence of UDP(^{14}C)Gal and 0.5 mg/ml of albumin. After extraction of the lipids (2.3.2) and the separation by thin layer chromatography (2.5.1), spots with radioactivity were identified with a Desaga thin layer scanner model 12-2 and scraped off. The silicagel was eluted with CCl_3H:CH_3OH=2:1 (v/v). The M(^{14}C)GDG was kept in this solution under N_2 at -20^{0}C.

2.2.1 *Isolation of chloroplast envelopes according to Poincelot and Day*

120 g of washed, deveined spinach leaves were homogenised in an Atomixer in 240 ml buffer, containing 0.2 M sucrose and 0.1 M tricine/NaOH pH 6.2, by mixing twice at full speed for 15 seconds. The homogenate was filtered in a hydraulic press through 8 layers of gauze with a maximum pressure of 100 bar.
The homogenate was centrifuged for two minutes at 5 000 m/s^2 in a Sorvall HB-4 rotor to remove cell debris and nucleic material. The supernatant was centrifuged again in the same rotor for one minute at 20 000 m/s^2. The supernatant was discarded and the pellet, containing intact and broken chloroplasts, was swollen in 25 ml 0.05 M tricine/NaOH buffer pH 7.4 for 20 minutes. The treatment was finished by two passes in a Potter homogeniser and the chloroplast suspension was adjusted to about 0.35 M sucrose by addition of 7.5 ml 1.5 M sucrose in 0.05 M tricine/NaOH buffer pH 7.4. The chloroplast suspension was laid on top of a discontinuous sucrose gradient, buffered as mentioned above. The gradient consisted of 10 ml 0.9 M sucrose and 10 ml 0.63 M sucrose in a Spinco SW 27 cellulose nitrate tube. Per experiment four tubes were centrifuged for 60 minutes at 1 000 000 m/s^2 (at R_{max}) in a Beckman model L2 preparative ultracentrifuge. Two pale, milky white bands were visible at the sucrose interfaces and a thick green pellet at the bottom of the tube (fig. 2.1). A long needle was brought carefully into the tube until its opening was two millimeters from the thylakoid pellet. The gradient was removed slowly by

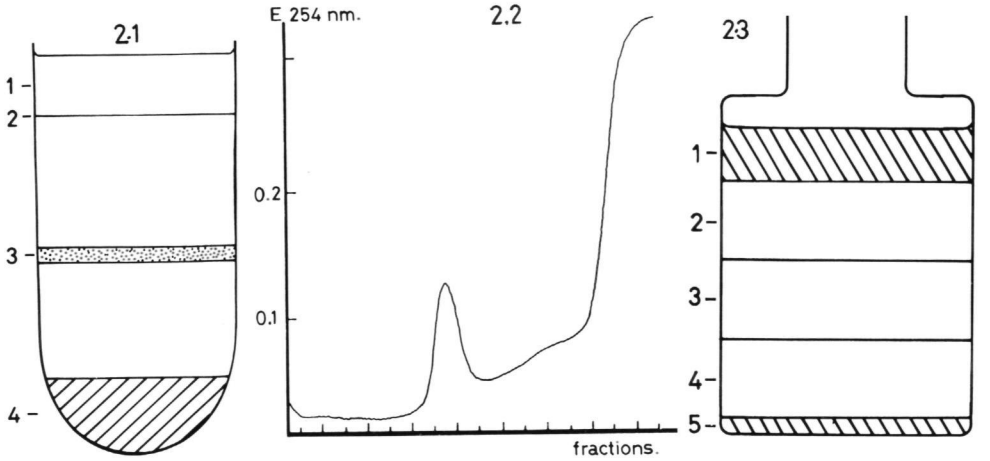


Fig. 2.1 Discontinuous sucrose gradient for the isolation of envelopes. *1* represents the stroma fraction, *2* the incomplete envelopes. This fraction is only present in the procedure of Poincelot and Day (1974). *3* represents F_2, the complete envelopes, and *4* the thylakoid material.

Fig. 2.2 Extinction profile of the gradient shown in fig. 2.1. The small peak in the middle indicates the presence of the complete envelopes, whereas the light envelope fraction is invisible because of the absorption by the stroma.

Fig. 2.3 Discontinuous sucrose gradient for the isolation of chloroplasts. Crude chloroplasts (*1*) were laid on top of a gradient consisting of 0.75 M (*2*), 0.91 M (*3*) and 1.5 M sucrose (*4*). After centrifugation (2.2.2) the pure chloroplasts were isolated in (*3*). Broken chloroplasts could be found in (*2*) and cell debris together with nucleic material in (*5*).

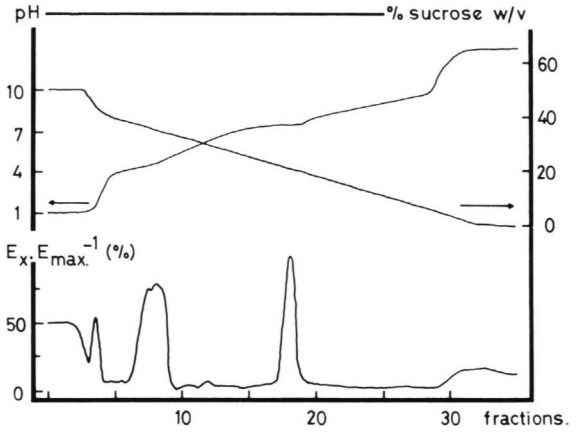


Fig. 2.4 Fractionation of an IEF gradient. After the IEF procedure as described in 2.2.3 the gradient was drawn and the extinction measured continuously. Sucrose content and pH were measured in each fraction. The peak in fraction 4 marks the beginning of the gradient and the end is indicated by an increase in absorption in fraction 30.

a LKB peristaltic pump. Its absorption was measured at 280 nm with an Uvicord II and divided into portions by a LKB fraction collector (fig. 2.2). The fractions containing envelope material were diluted to 0.33 M sucrose with tricine/NaOH buffer pH 7.4 and centrifuged in a Beckman 50 Ti rotor for 90 minutes at 1 000 000 m/s^2 (at R_{max}). The pellets were resuspended in a two ml Potter homogeniser and pooled into two fractions, (F_1 and F_2) according to fig. 2.1.

2.2.2 *Isolation of envelopes according to Joyard and Douce*

1.5 kg of spinach leaves from the market, or 900 g from the greenhouse were washed, dried and deveined. 700 g of laminae remained in either case and were divided in 100 g portions. Each portion was homogenised in 200 ml buffer 1 (containing 0.2 M sucrose, 0.1 M tricine/KOH buffer pH 7.2 and 1 g albumin per litre), in an Atomixer at full speed for 15 seconds. The homogenate was filtered through 8 layers of gauze in a wide funnel and the filtrate was centrifuged in a Sorvall RC5 centrifuge in a HS4 rotor with 250 ml buckets for one minute at 20 000 m/s^2 (at R_{max}). The supernatant was discarded and the pellet, containing cell debris, nucleic material and intact and broken chloroplasts was resuspended in 40 ml buffer 1 but without albumin. This crude chloroplast preparation was purified on a discontinuous sucrose gradient consisting of 50 ml 1.5 M sucrose, 75 ml 0.92 M sucrose and 50 ml 0.75 M sucrose, all buffered with 0.1 M tricine/KOH buffer pH 7.2. The gradient was centrifuged for 15 minutes at 8 000 m/s^2 (at R_{max}) in the Sorvall rotor mentioned above. The 0.92 M sucrose layer was collected (fig. 2.3) and the chloroplasts were carefully diluted with an equal volume of buffer 1 without albumin under continuous stirring. The mixture was centrifuged in the HS4 rotor for 10 minutes at 22 000 m/s^2 (at R_{max}). The supernatant was discarded and the intact purified chloroplasts were shocked osmotically by addition

of 20 ml of 20 mM tricine/KOH buffer pH 7.2, which contained also 5 mM $MgCl_2$. The pellet was resuspended quickly with a 10 ml pipette and 7 ml of the chloroplast suspension were laid on a buffered sucrose gradient consisting of 5 ml 0.92 M sucrose and 5 ml 0.6 M sucrose in 0.1 M tricine/KOH buffer pH 7.2, containing 10 mM $MgCl_2$. The gradients were centrifuged in a SW27 rotor in a Beckman L2 preparative ultracentrifuge at 1 100 000 m/s^2 (at R_{max}) during 60 minutes. After centrifugation there were two major differences in comparison to the gradient of Poincelot and Day (fig. 2.1). The stroma fraction was coloured straw-yellow in the procedure according to Joyard and Douce, whereas in the procedure of Poincelot and Day employed previously it was colourless. This is probably the result of a much higher concentration of chloroplasts on the gradient. Secondly, instead of two pale bands only the F_2 fraction was present now and this fraction was coloured bright yellow. The yellow band was removed carefully with a pipette and diluted with buffer 2 (containing 0.1 M tricine/KOH pH 7.2 and 10 mM $MgCl_2$) to about 0.3 M sucrose, which was checked with a refractometer. The envelope fraction was centrifuged in a SW27 or SW40 rotor in a Beckman ultra-centrifuge for one hour at 1 100 000 m/s^2 (at R_{max}). By diluting to only 0.3 M sucrose contamination by stroma material was minimised. The bright yellow pellets, containing about 1.4 mg of proteins were resuspended in 2.8 ml buffer 2 by sonification at 65 mW for 30 seconds with a Megason ultrasonic disintegrator operating at 20 kHz. At various stages of the isolation procedure the intactness of the chloroplasts can be checked for by phase contrast microscopy (Lilley *et al.* 1975). The pigments of isolated envelopes can be extracted with ethanol (Wintermans and De Mots 1965) or with acetone (Arnon 1949) to check for contamination by chlorophyll from thylakoid material. The whole procedure was performed at temperature between 0^0C and 5^0C.

2.2.3 *Isolation of envelope subfractions*

Envelope membranes were isolated according to 2.2.1 and the pellet resulting from the last centrifugation step (F_2) was resuspended in distilled water by sonification (1 minute, 75 mW). The envelope suspension, together with 45 ml distilled water was mixed with 54 ml 1.35 M sucrose to form a continuous gradient in an iso electrical focussing column from LKB (Bromma, Sweden). For the formation of a pH gradient 0.03% ampholines (pH range 3.5-10) were added to the solutions mentioned above. The cathode solution consisted of 10 ml 0.25 M NaOH and for the anode the solution consisted of 20 ml 0.16 M H_3PO_4 which contained also 1.7 M sucrose. The separation was effected by application of 300 volt for 16 hours with a constant voltage source (LKB). During the IEF procedure, in which the column was cooled to 4^0C with a Kryostat cooling system the current decreased from about 4 to 1 mA. After 16 hours the electricity was cut off and the gradient drawn with a LKB peristaltic pump through an absorption measuring cell (LKB Uvicord 2). Fractions which exhibited absorption at 280 nm (fig. 2.4) were diluted to 0.3 M sucrose with 0.1 M tricine/NaOH buffer pH 7.4 and centrifuged for 1 hour at 1 100 000 m/s^2 (at R_{max}) in a Beckman L2 preparative ultra-centrifuge. The pellets were resuspended in 0.1 M tricine/NaOH buffer pH 7.4.

2.3 ANALYSES

2.3.1 *Proteins*

Proteins were analysed quantitatively by the method of Lowry *et al.* (1951) in comparison to a bovine serum albumin standard.

2.3.2 *Lipids*

Lipids were extracted by a method based on the Bligh and Dyer extraction. To 350 µl of incubation mixture 1.5 ml CH_3OH was added. After mixing on a Vortex whirlmixer 1.5 ml $CHCl_3$ was added and the sample was mixed again. Phase separation was accomplished by addition of 1 ml H_2O. Emulsions were broken by centrifugation for 3 minutes at 10 000 m/s^2 (at R_{max}) in a Sorvall GS centrifuge. The upper layer containing the H_2O and CH_3OH was removed with a small pipette and a vacuum pump. The chloroform layer was washed twice with the upper phase from a mixture of 0.5 l CH_3OH, 0.5 l $CHCl_3$ and 0.45 l H_2O. Finally the $CHCl_3$ layer was taken to dryness under N_2 and the lipids were redissolved in 25-50 µl $CHCl_3$:CH_3OH=10:1. The separation was performed by thin layer chromatography (see 2.5.1).
Galactolipids were identified by spraying the plate with the periodate Schiff reagent (Kates 1972), after which they were coloured pink red on a colourless background developing into purple spots on a pink background.
Phospholipids were identified by spraying with the Dittmer and Lester reagent (Kates 1972). Phosphorus containing compounds appear as blue spots on a white background.
All spots containing organic material can be visualised after spraying with 25% H_2SO_4 and subsequent heating of the plates to 120°C for 15 minutes. Sterols appear as reddish brown spots, free fatty acids, and mono-, di- and triacylglycerols are yellow brown and other compounds give dark brown spots. However, after some time all colours faded into brown black. Reheating of the plates brought back the original colours to some extent.
For quantitative analysis the spots were made visible by I_2

vapour, which was removed in vacuo, after all spots had been marked with a pencil. Quantitative estimation of acyllipids is described in 2.5.2.
Quantitative analysis of galactolipids was performed, after separation of the lipids by thin layer chromatography, by colorimetric determination of galactose with the anthrone reagent according to Yemm and Willis (1954). Phospholipids were determined according to Rouser *et al.* (1970) after degradation of the organic material with 70% $HClO_4$ at 160°C for 1 hour.

2.3.3 *Pigments*

Pigments were separated by thin layer chromatography (2.5.1) if required. The chlorophyll content was measured in a 96% ethanolic extract according to Wintermans and De Mots (1965) without further purification. For this determination a complete spectrum was made or, routinely, the absorbance was measured at 649 and 665 nm with a Beckman model 25 double beam spectrophotometer.
In a few cases the chlorophyll content was determined in a 80% acetone extract according to Arnon (1949).

2.3.4 *Radioactive label*

Labelled lipids were extracted according to Bligh and Dyer (1959) (2.3.2) and separated by thin layer chromatography (2.5.1). Radioactivity was spotted with a Desaga thin layer scanner model 12-2 or by autoradiography with Kodirex X-ray film (Kodak), which was exposed for 5 days at room temperature. The Desaga scanner could resolve spots with more than 500 dpm ^{14}C or 25 000 dpm ^{3}H. With an exposure of 5 days the X-ray film could resolve spots with about 40 dpm ^{14}C, but ^{3}H was hardly detectable even when over 200 000 dpm were present in a spot. Spots with

radioactivity on the thin layer plates were marked with a pencil and scraped off into counting vials, Lipoluma (Baker) was added and the label was counted in a Philips PW4540 liquid scintillation analyser.
For quantitative determination of the incorporated label the Bligh and Dyer extract was transferred into a counting vial and taken to dryness with a stream of air. Lipoluma was added and the vials were counted as mentioned above. If pigments absorbing in the ultra violet region were present quenching was minimised by irradiation of the chloroform solution with ultra violet light. Labelled UDPGal and other polar compounds were transferred into counting vials in water or methanolic solution and counted after addition of Aqualuma (Baker).

2.3.5 *Enzymic activities*

The ATPase activity of isolated envelopes was measured by quantification of the inorganic phosphate liberated from ATP. The free phosphate was determined by the methods of Fiske and Subbarow (1925), Tausky and Shorr (1953) and Lowry and Lopez (1946), of which the latter gave the most satisfying results. The reaction was stopped by addition of 0.1 ml icecold 34% CCl_3CO_2H to 0.6 ml incubation mixture. The mixture was cooled in ice for 10 minutes and then centrifuged in a Sorvall SS34 rotor at 200 000 m/s^2 (at R_{max}) for 20 minutes. From the supernatant 0.5 ml was taken and added to 0.5 ml 0.4 M CH_3CO_2Na. Then 0.1 ml 10% vitamine C and 0.1 ml 2.5% amoniummolybdate in 3 M H_2SO_4 were added and the mixture was incubated at 45^0C for 5 and 10 minutes. The absorption was measured at 810 nm and extrapolation to 0 minutes resulted in a measure for the amount of phosphate liberated.
Galactosyltransferase and lipase activities were measured with radioactively labelled substrates or products (2.3.4). The reaction:

UDPGal + diacylglycerol → UDP + MGDG

was analysed also with ^{31}P NMR (2.6).
NAD(P)H-cytochrome c oxidoreductase activities were determined by spectral changes at 550 nm according to Douce *et al.* (1973b). Peroxidase and catalase activities were measured according to Lück (1963). Changes in absorbance at 470 and 240 nm respectively were monitored continuously with a Beckman double beam spectrophotometer in thermostated cuvettes (30°C).

2.4 REACTION MIXTURES

2.4.1 *Incubations for quantitative determination*

Usually these incubations were carried out with UDP(^{3}H)Gal, and the total incorporation was measured. A standard incubation mixture had the following composition: envelope material equivalent to 100 µg/ml protein, 0.1 M tricine/KOH buffer pH 7.2, 10 mM $MgCl_2$ and 0.2 mM UDPGal ($5 \cdot 10^{12}$ cpm ^{3}H/mol), all final concentrations. The volume was 250 µl and the incubations were carried out at 30°C for 5 minutes. The incubation was started by addition of preincubated envelopes (5 minutes at 30°C) to the rest of the incubation mixture, which was preincubated similarly. The reaction was stopped by addition of 1.5 ml CH_3OH and 1.5 ml $CHCl_3$ and mixing on a Vortex. All effectors were dissolved in 0.1 M tricine/KOH buffer and the pH was adjusted to 7.2. Oleic acid was added as 1 µl ethanolic solution. If incubations were performed at a pH different from 7.2, this was accomplished by addition of 1 volume of 1 M buffer of the pH desired, using acetate for pH values below 7 and tris for pH values above 7.

2.4.2 *Incubations for qualitative determination*

The volume of the incubation mixture was increased to 350 or 500 µl in order to compensate the lower specific activity of UDP(^{14}C)Gal and to obtain sufficient activity in some of the minor fractions. Final concentrations of protein, substrate and buffer were identical to those mentioned in 2.4.1. The specific activity of UDPGal was 10^{12} cpm ^{14}C/mol. When the concentration of a component differs from those mentioned, it is stated in the experiment. If incubations were carried out for 30 minutes on more, the incubation mixture was prepared in ice and the reaction was started by transferring the test tube to a waterbath of 30°C. However, incubations for Arrhenius plots were always preincubated for 5 minutes as mentioned in 2.4.1. The reactions were stopped by addition of 1.5 ml CH_3OH, mixing, addition of 1.5 ml $CHCl_3$ and subsequent mixing.

2.4.3 *Incubations for NMR*

References for NMR spectra were UDPGal and UDP. Both were dissolved to a concentration of 10 mM in a mixture containing 0.1 M tricine/KOH buffer pH 7.2, 10 mM $MgCl_2$, 1 mM EDTA and 50% D_2O. The incubation mixture for NMR had the following composition: envelope membranes equivalent to 1 mg protein/ml, 10 mM $MgCl_2$, 10 mM UDPGal, 0.1 M tricine/KOH buffer pH 7.2, 1 mM EDTA and 50% D_2O. After incubation of 400 µl of this incubation mixture for 60 minutes at 30°C, this was cooled in ice and spectra were accumulated at 4°C. For the determination of the stability of UDPGal, a 10 mM solution as described above was kept 4 days at room temperature.

2.4.4 *Incubations for ATPase*

Envelope material equivalent to 50 µg protein, 2 mM ATP, 0.5 M tricine/NaOH buffer pH 7.4 and 8 mM $MgCl_2$ were incubated in a volume of 500 µl for 20 minutes at 30°C. The reaction was started by addition of the envelope material and stopped by addition of 100 µl icecold 34% CCl_3CO_2H.

2.4.5 *Incubations for NAD(P)H-cytochroom c oxidoreductase*

Reaction mixtures contained, in 1 ml total volume: 1 µmol NAD(P)H, 0.1 µmol cytochroom c, 0.5 µmol KCN, 50 µmol phosphate buffer pH 7.2 and 0.1 to 1 mg protein. The incubation was carried out in a thermostated cuvette at 30°C and started by addition of envelope material.

2.4.6 *Incubations for catalase and peroxidase*

Envelope material equivalent to 0.1-1 mg protein was incubated at 30°C in the presence of 0.1 M acetate buffer pH 5 for peroxidase and pH 7.4 for catalase and 20 mM H_2O_2 in a total volume of 1 ml. When peroxidase activity was to be measured guaiacol was added to a final concentration of 20 mM. The reactions were started by addition of hydrogen peroxide.

2.4.7 *Albumin washed membranes*

Albumin washed membranes were obtained by incubation of envelope membranes for 20 minutes at 30°C with buffer 2 containing also 2.5 mg albumin/ml. Subsequently the envelopes were centrifuged at 4°C in a SW 56Ti rotor in a Beckman ultracentrifuge for 30 minutes at 1 000 000 m/s^2 (at R_{max}). The pellet was resuspended by light sonification (50 mW during 30 s) in buffer 2 and centrifuged again to remove all albumin. The pellet was resuspended in the desired medium.

2.4.8 *Pulse labelled membranes*

Pulse labelled membranes were obtained in two ways. Individual portions of membranes were pulse labelled by preincubation with arbitrary amounts of UDP(^{14}C)Gal in a volume of 175 µl for 20 minutes at 30°C and subsequent centrifugation for 15 minutes at 1 000 000 m/s^2 (at R_{max}) in a Beckman Airfuge. After removal of the supernatant the pellets were resuspended in the chosen medium. Larger batches of membranes were incubated with UDP(^{14}C)Gal for 20 or 30 minutes, cooled on ice and centrifuged in a SW 56Ti rotor at 4°C for 30 minutes at 1 000 000 m/s^2 (at R_{max}). The pellet was resuspended in the desired medium and divided into portions.
If labelled acylMGDG was the component of major interest, the membranes were incubated at 30°C at pH 6 and if MGDG was of major interest, the mixture was incubated for about 30 minutes at pH 8.0 or pH 9.0.

2.5 CHROMATOGRAPHY

2.5.1 *Thin layer chromatography*

Galactolipids were separated on Kieselgel 60 thin layer plates from Merck, Darmstadt, which were activated for 20 minutes at 110°C not more than 3 hours before use. The spots were applied with a 50 µl Hamilton syringe from a $CHCl_3$:CH_3OH=9:1 solution prepared as described in 2.3.2. During application the solvent was evaporated with a stream of warm air to keep the spots small and to accelerate the procedure. The eluent used for separation of radioactively labelled galactolipids was $CHCl_3$:CH_3OH:H_2O=65:25:3.5 (v/v) (Allen and Good 1971). The decomposition of UDPGal was checked by application of 5 µl of the 2 mM stock solution on a cellulose thin layer plate from Merck, Darmstadt, which was eluted with 0.2 M NH_4HCO_3. The spots were localised with ultra violet light or with a Desaga thin layer scanner.
For gas liquid chromatography the lipids were separated with thin layer chromatography using three elution runs. In the first and second elutions galacto- and phospholipids were separated and a third elution was introduced to separate pigments from diacylglycerols, acylMGDG and free fatty acids. Polar lipids did not move in this third development. Kieselgel 60 plates were activated as described above. Envelope lipid extracts were made as described in 2.3.2 and applied as mentioned above. In the first run the plate was eluted with CH_3COCH_3:C_6H_6:H_2O=91:30:8 (v/v) (Pohl *et al.* 1970). The plate was dried in vacuo at 30°C for 30 minutes and developed with $CHCl_3$:CH_3OH:7 M NH_4OH=65:35:4 (v/v) in a direction perpendicular to the first. The plate was dried in the same way as mentioned before and eluted, in opposite direction to the second run, with petroleum ether 40/60: $CH_3CH_2OCH_2CH_3$:HCO_2H=60:40:1.5 (v/v). The plate was dried and the spots were made visible by I_2 vapour.

2.5.2 *Gas liquid chromatography*

To spots obtained as mentioned above, heneicosanic acid (C_{21-0}) was added, as an internal standard for quantification. The spots were scraped off of the chromatogram and transesterified by incubation at 70°C for 2.5 hours with 5 ml 5% H_2SO_4 in CH_3OH. After cooling the esters were extracted with petroleum ether (boiling range 35-47°C)(extra pure, Baker), neutralised with $NaHCO_3$ and evaporated in a stream of N_2. The lipids were dissolved in a small volume of CS_2 and injected in a column packed with 10% SP 2330 on chromosorb WAW 100-120 (Supelco) in a Varian aerograph series 2700. The column temperature was 190°C, the carrier gas, N_2, had a flow rate of 40 ml/minute and material was measured with a flame ionisation detector. For quantitation, the product of peak height times retention time was calculated (Carrol 1961).

2.6 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

NMR was monitored with a Varian X L 100 apparatus operating at 40.5 MHz at the Fourier Transform mode. The chemical shifts in the proton decoupled ^{31}P-NMR spectra were measured relative to 30% H_3PO_4 in D_2O. All solutions were measured for 1 h at 4°C and contained 50% D_2O.
The experiments were performed at the Department of Biophysical Chemistry of the Catholic University Nijmegen.

2.7 DIFFERENTIAL SCANNING CALORIMETRY

D.S.C. was performed at the Laboratory of Biochemistry of the State University, Utrecht with a Perkin-Elmer DSC-2B apparatus operating at a heating rate of 5^{0}C/minute.

3 SITE OF GALACTOLIPID FORMATION

3.1 INTRODUCTION

As we have seen in the first chapter, the incorporation of galactose from UDPGal into galactolipids was described first by Benson *et al.* (1958) with the scheme:

UDPGal + diacylglycerol → MGDG + UDP I

In 1964 Neufield and Hall localised the enzyme catalysing (1) in the chloroplasts of spinach leaves. More recently Douce (1974) showed that the site of galactosylation in spinach chloroplasts was the envelope. This was soon confirmed in our laboratory by van Hummel *et al.* (1975), although they did not investigate the absence or presence of various marker enzymes in the preparation.

3.2 PURITY OF ISOLATED ENVELOPES

In agreement with the results of Poincelot and Day (1973) and Douce *et al.* (1973a) no microsomal, mitochondrial, cytoplasmatic or stromal contamination could be detected by measuring activities of peroxidase, catalase and NAD(P)H-cyt.c oxidoreductase.

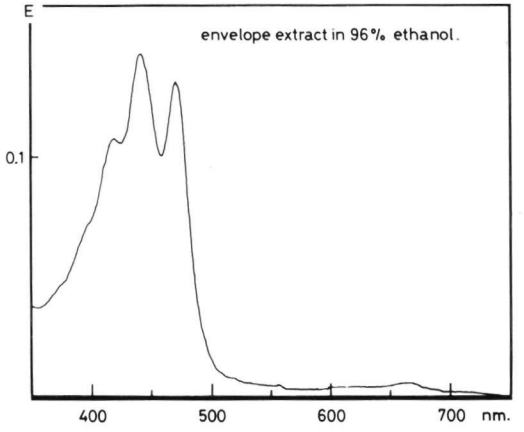


Fig. 3.1 Absorption spectrum of envelope lipids. Envelope material was extracted with 96% ethanol and the absorption was measured against a blanc of 96% ethanol with a Beckman double beam spectrophotometer. The presence of violaxanthin, lutein and/or neoxanthin is demonstrated by the small peak at 410 nm.

Furthermore the absence of thylakoid material could be shown by spectrophotometric analysis of a lipid extract in 80% acetone (Arnon 1949) or in 96% ethanol (Wintermans and De Mots 1965) (fig. 3.1).

In isolated envelopes 2 marker enzymes were shown to be present. A Mg^{2+} dependent nonlatent DCCD-insensitive ATPase with activities reported up to 100 µmol/mg.h (Poincelot 1976). The second marker enzyme is the galactosyltransferase for which a maximum velocity was reported of 2.7 µmol/mg.h (Joyard and Douce 1976b). Both marker enzymes were present in our envelope preparation. We were never able, however, to find the high activities of ATPase mentioned above, the maximum activity being about 5 µmol/mg.h. This is in agreement with the findings of Joyard and Douce (1976b), who reported a maximum velocity of about 7 µmol/mg.h.

For the galactosyltransferase the incorporation velocities agreed well with those mentioned above, although these velocities depend strongly on the incubation circumstances as will be discussed in

chapter 5.
In addition we were able to demonstrate the absence of UDPG epimerase in our envelope preparations. The presence of this enzyme in the cytoplasm has been reported by Bird *et al.* (1974). When we incubated isolated envelopes for 30 minutes in the presence of equal amounts of UDP(^{14}C)G (10^{12} cpm/mol) and UDP(^{3}H)Gal ($4 \cdot 10^{10}$ cpm/mol), we could find no ^{14}C-label in the galactolipids that were formed in this reaction. This demonstrates both the absence of epimerase activity as well as the high specificity for galactose of the galactosyltransferase.

3.3 LOCALISATION OF GALACTOSYLTRANSFERASE ACTIVITY WITHIN THE ENVELOPE

It is a tempting suggestion that the final step in synthesis of galactolipids, the coupling of galactose to diacylglycerol takes place in the inner envelope membrane, since lamellae are believed to arise from protrusions of the envelope (Sitte 1977). This inner membrane is reported to be active in metabolism and compartimentation (Heldt and Rapley 1970). The outer membrane, which is permeable to small molecules (Sprey and Laetsch 1975) carries a strong negative charge at pH 7.3 (Westrin *et al.* 1975, Dubacq 1977) and has predominantly immunological properties (Billecocq 1975). IEF techniques seemed a feasible approach in trying to obtain a separation of inner and outer envelope membranes.
Envelopes were mixed through a sucrose gradient and the voltage was applied. After 16 h this resulted in a separation into 2 fractions (fig. 3.1). The major fraction was collected at pH 7 and contained both marker enzymes. ATPase activity was measured at one substrate concentration and no pertinent increase in

enzyme activity could be observed. Galactosyltransferase activity was measured at various substrate concentrations and plotted according to Lineweaver and Burk (1934). In comparison to unfractionated envelopes both V_{max} and K_m were increased. However, since this effect may also be induced by a change in fatty acid content (see chapter 4), the incubation should be repeated in the presence of albumin in order to learn if really an increase is obtained in the relative amount of enzyme in the envelope subfraction. A minor fraction was collected at pH 4 which means that at physiological pH it is negatively charged. The protein content of this fraction was rather variable and the fraction contained neither marker enzymes. We have not succeeded in characterising this fraction, although we tried the following techniques: electron microscopy, polyacrylamidegel electrophoresis of the extracted proteins and thin layer chromatography of the lipids. Hence the nature and origin of this fraction remains obscure.

Neuburger *et al.* (1977) report the separation of envelopes into 2 unequal fractions by ultracentrifugation after addition of cytochrome c. It was not possible, however, to obtain in this way a reproducible difference with respect to composition and enzymic activities between the 2 subfractions (Douce personal communication). Hence one can conclude also that these experiments did not result in the separation of inner and outer envelope membranes.

4.1 INTRODUCTION

As we have seen in the previous chapter, Benson *et al.* (1958) introduced a reaction for the biosynthesis of galactolipids. This scheme was confirmed with respect to the substrate UDPGal by Neufield and Hall (1964). They reported that label was incorporated from UDP(^{14}C)G into lipids by spinach chloroplast extracts. Analysis learned that the lipids contained labelled galactose and no glucose, from which the authors concluded that UDPG had to be converted into UDPGal prior to glycolipid formation.

Joyard and Douce (1976a,b) demonstrated the presence of great amounts of diacylglycerol in isolated envelopes from spinach chloroplasts. Upon addition of UDPGal they observed a decrease in diacylglycerol content and a concomitant increase in the amount of MGDG. This too, can be regarded as a support for the hypothesis of Benson *et al.* (1958).

In isolated envelopes the galactosyltransferase is retained in a much better condition than, for example, in chloroplast preparations after acetone precipitation. This resulted in incorporation velocities that were high enough to monitor the amount of UDPGal that is transferred by the enzyme and to test the validity of the hypothesis of Benson *et al.* (1958) with ^{31}P NMR. Spectra were made of UDPGal, UDP and of a mixture of UDPGal and envelope material after an incubation of 1 hour at

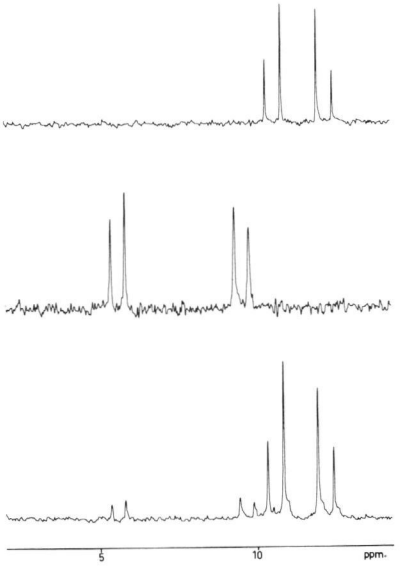


Fig. 4.1 ^{31}P NMR spectra of UDPGal (top), UDP (centre) and incubation mixture. For experimental details see sections 2.4.3 and 2.6.

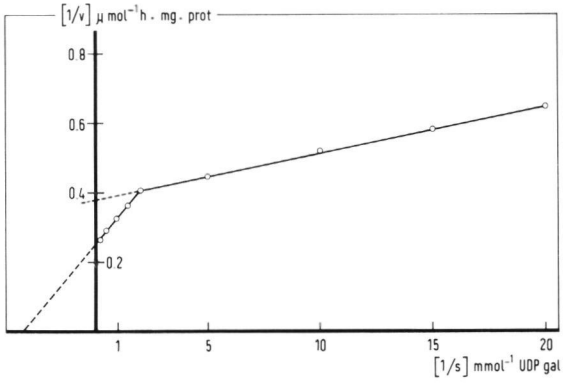


Fig. 4.2 Lineweaver-Burk plot of UDPGal-diacylglycerol galactosyltransferase On the abscissa is plotted the reciprocal value of the substrate concentration in mM^{-1} , on the ordinate the reciprocal reaction velocity in μmol^{-1} ·h·mg protein. The reaction mixture was incubated for 7 minutes at 30^0C. Total lipid incorporated radioactivity was used for calculation of enzyme activity. K_m and V for the high affinity site were calculated to be: 34 μM UDPGal and 2.7 μmol/h·mg protein, respectively. For the steep part of the plot K_m was found to be 310 μM and V 3.85 μmol/h·mg protein.

30°C. From fig. 4.1 it is obvious that during the incubation UDP appears. When incubated without envelope material UDPGal very slowly decomposes to monophosphorous compounds without the appearance of detectable amounts of UDP. These monophosphorous compounds were not further characterised but presumably represented galactose-1-phosphate and UMP, which had identical spectra. From an incubation with UDP(^{14}C)Gal the amount of incorporated (^{14}C)galactose was calculated with the same batch of envelopes and was found to agree very well with the amount of UDP that was liberated according to the NMR spectrum. In our opinion this is a convincing argument that UDPGal is a substrate in galactosylation resulting in UDP as one of the products of the reaction in agreement with reaction scheme I.

4.2 COMPETITIVE INHIBITION

After an incubation of envelope membranes in the presence of UDP(^{14}C)Gal during 5 minutes or less, label is found almost exclusively in MGDG, the total of other labelled compounds being less than 5% (Joyard and Douce 1976b) (fig. 4.2). This incorporation can be described very accurately according to the theory of enzyme kinetics of Michaelis and Menten (Lehninger 1975) and, therefore, valuable information can be gathered from Lineweaver-Burk plots. Fig. 4.2 shows that at a high substrate concentration an extra activity is developed by the enzyme with a low affinity towards UDPGal. By analogy to the interpretation of the kinetic experiments of Kang *et al.* (1977) one may suggest that this points to allosteric stimulation of the transferase by binding of a second molecule of UDPGal to the protein. Since the affinity of this second enzymic site for

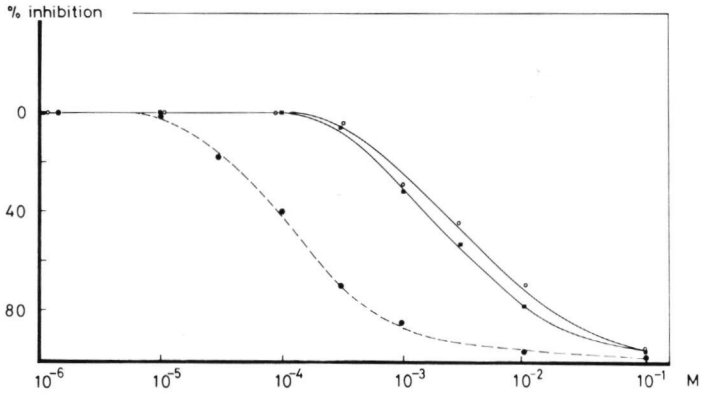


Fig. 4.3 Dose-effect curves of uridine nucleotides. On the ordinate the percentage inhibition is plotted, and on the abscissa the concentration of UMP or UDPG (■), UDP (●, dashed line) and UTP (○). Envelope material was incubated with 0.2 mM UDPGal plus the indicated concentrations of effector, for 5 minutes at 30°C and at pH 7.2. 50% Inhibition required a concentration of either 0.13 mM UDP, 2.5 mM UMP or UDPG, or 4 mM UTP.

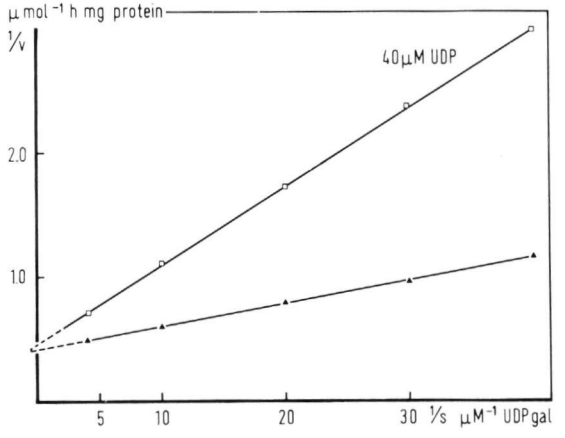


Fig. 4.4 Inhibition of galactose incorporation by UDP. Reciprocal values of UDPGal concentration (in mM^{-1}) are plotted on the abscissa, on the ordinate the reciprocal reaction velocities are given for a reference incubation (▲) and an incubation with 40 μM UDP (□). The reaction was started by addition of 25 μg envelope proteins to a final volume of 250 μl. After 5 minutes incubation at 30°C the reaction was stopped by addition of 1.5 ml CH_3OH.

UDPGal is so low, the K_m being about 350 µM instead of 45 µM for the first binding site, this second enzymic site is probably not important for enzyme activity under physiological circumstances.
We tried to influence reaction velocities by addition of substrate analogues to the reaction mixture. Galactose as well as galactose-1-P and glucose-1-P appeared to be without any effect up to a final concentration of 10 mM. Also uridine and uracil had no effect on incorporation velocities. Uridine nucleotides on the other hand proved to be inhibitory (fig. 4.3). They were shown to inhibit the reaction competitively as demonstrated by the Lineweaver-Burk plot of UDP in fig. 4.4., in contrast to other nucleotides such as ADP, AMP, CDP, CMP and GTP which were ineffective even at a final concentration of 10 mM. ATP had variable effects which, however, did not seem relevant in this context, and will be discussed in section 4.6. UDP was the most potent inhibitor of the nucleotides, whereas UTP is even less effective than one would read from fig 4.3, since most of the inhibition is due to contamination of the UTP by UDP. According to the manufacturer UTP contains about 4% UDP. This means that at a calculated concentration of 2.5 mM UTP, which results in a 50% inhibition of the reaction, the actual UDP concentration is about 0.1 mM which accounts for 40% inhibition. So only 10% inhibition results from about 2.4 mM UTP. UMP inhibits the reaction for 50% at a concentration of 2 mM. Under the circumstances of fig 4.3, that is at 0.2 mM UDPGal, UDP inhibits the reaction for 50% at a concentration of 0.13 mM; thus the affinity of the enzyme for UDP is higher than for UDPGal. Calculations of K_m and K_i for UDPGal and UDP respectively from fig. 4.4 lead to the same result: K_m is 43 µM and K_i for UDP can be calculated to equal 18 µM. This leads to the conclusion that UDP is the part of the substrate molecule most important for its recognition by the enzyme.
However, the sugar residue is also important as can be seen from the the inhibition of UDPG (fig. 4.3). UDPG is also a competitive inhibitor with an affinity equal to that of UMP (K_i is ±100 µM).

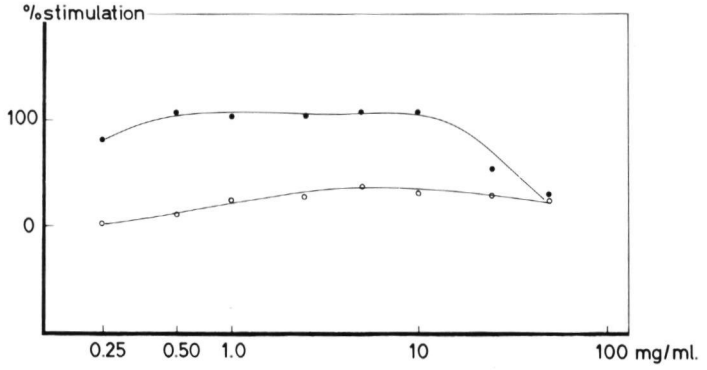


Fig. 4.5 The effect of albumin on the incorporation velocity (●) and on the percentage of label in DGDG and T(e)GDG (o). On the ordinate is plotted the percentage stimulation in comparison to a reference incubation without albumin (from the same batch of envelopes). The final concentration of albumin is plotted on the abscissa. The incubations were carried out at 30°C for 5 minutes (●) or for 1 h (o) as described in 2.4.1 and 2.4.2.

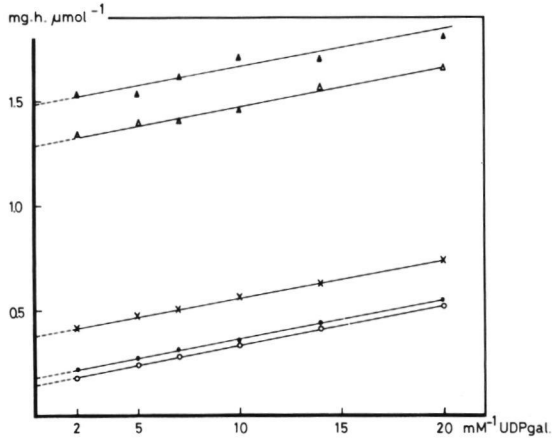


Fig. 4.6 The effects of oleic acid and albumin on UDPGal-diacylglycerol galactosyltransferase. In the graph the reciprocal reaction velocities (ordinate) are plotted against the reciprocal UDPGal concentrations (abscissa) for: (from top to bottom) envelopes in the presence of 150 µM oleic acid (▲), albumin washed envelopes in the presence of 150 µM oleic acid (Δ), envelopes (×), albumin washed envelopes (●) and membranes in the presence of 1.5 mg albumin/ml (o). Incubations were carried out at 30°C for 5 minutes as described in 2.4.1. From the graph the content of free fatty acids of these envelopes was calculated to be equivalent to 30 µM oleic acid.

UDPG resembles UDPGal very much, the only difference being the hydroxyl group at the C_4-atom of the sugar, which is equatorial in glucose, whereas it is axial in galactose. Nevertheless the affinities for the enzyme differ by a factor 2. Since the affinity for UDPGal is lower than for UDP it is very likely that the sugar moiety has a negative influence on the binding to the enzyme which means that UDPG must be actively rejected by the enzyme as soon as the glucose moiety is recognised.
The result of the enzymic action on the substrate UDPGal are the products UDP, which is released into the medium, and the galactose molecule bound to the enzyme or to a prosthetic group. The next step then may be the attachment of the galactose to a diacylglycerol molecule in a β-glycosidic way.

4.3 UNCOMPETITIVE INHIBITION

It has been known for a long time (Helmsing and Barendse 1970) that albumin enhances the incorporation of label from UDP(^{14}C)Gal into lipids. A dose-effect curve of this enhancement (fig. 4.5) shows that the effect is not specific because a wide range of albumin concentrations results in the same stimulation of the reaction velocity. In experiments with different batches of spinach the enhancement varied from 100% to 300% stimulation but the concentration range of albumin in which the enhancement was found showed very little variation. This also is an argument against a specific interaction.
Because the most common known effect of albumin is the binding of free fatty acids, we tried oleic acid as an inhibitor of the reaction (fig. 4.6). Oleic acid was indeed found to be an inhibitor, the inhibition being uncompetitive. Albumin enhanced the reaction also in an uncompetitive way. According to the

theories of enzyme kinetics of Michaelis and Menten (Lehninger 1975), this means that the inhibition by oleic acid occurs after formation of the enzyme-galactose complex and that oleic acid does not compete for the UDPGal binding site. Moreover, the uncompetitive enhancement by albumin shows that the isolated membranes were contaminated by free fatty acids. The variability of the effect shows that the quantity of free fatty acids can differ considerably in envelopes isolated from different batches of spinach. So it might be interesting to look at the effect of removal of the fatty acids by albumin which in turn is washed away before the incubation with UDPGal is started. In one experiment five portions were taken from the same batch of isolated envelopes and two portions of these were washed with albumin (2.4.5). Two washed and two unwashed portions were incubated in the presence of various concentrations of UDPGal with and without 150 µM oleic acid. Incorporation rates were plotted according to Lineweaver and Burk (1934). The fifth portion was incubated at the same UDPGal concentrations but in the presence of 1.5 mg albumin ml^{-1}. The results are presented in fig. 4.6. The top line represent the incubation of untreated membranes in the presence of oleic acid, the second line from the top the incubation of albumin washed membranes in the presence of the same amount of oleic acid, the third line the reference incubation of untreated membranes without effector. The second line from the bottom represents the incubation of albumin washed membranes without effector and the bottom line the incubation of untreated envelope membranes in the presence of albumin in the same concentration as was used to wash fatty acids out of the membranes. This leads to the following conclusions: a) washing with albumin is less stimulating than incubating in the presence of albumin, b) washing with albumin decreases the K_i of oleic acid considerably, according to fig. 4.6 from about 50 µM to 20 µM. From conclusion a) it can be inferred that the membranes still contain some fatty acids, which may arise both from ultracentrifugation effects as well as from lipase activities in the chloroplast envelopes. The observed

low K_i value strongly suggests that fatty acids do not inhibit by a general effect on the membrane but interact specifically with an enzymic site.

4.4 EQUILIBRIUM EXCHANGE

In 1977 Joyard and Douce showed that chloroplast envelopes could synthesize diacylglycerol from acyl-CoA and (^{14}C)glycerol-3-phosphate. Unexpectedly, they observed label in MGDG even before UDPGal was added. Upon addition of UDPGal the incorporation of label into MGDG was markedly enhanced and in their comments they neglected the label previously incorporated. Since this incorporation might be due to an exchangeability of diacylglycerol moieties of MGDG, we tried to reproduce these results from a different starting point. Envelope membranes were sonified with (^{14}C)dioleoylphosphatidylcholine (2.1.4) and at time zero phospholipase C was added and the mixture was transferred to a shaking waterbath at 30^{0}C. After various times aliquots were taken from the incubation mixture and analysed. The labelled diacylglycerol was indeed incorporated into MGDG although no UDPGal was present (fig. 4.7). Addition of UDP(^{3}H)Gal after 40 minutes with subsequent analyses after various incubation times justified our opinion that, firstly, the galactosyltransferase reacts as usual despite the addition of phospholipase C, as can be seen from incorporation of ^{3}H-label and, secondly, that the incorporation of fatty acyl label into MGDG is enhanced by addition of UDPGal. Moreover, it appears that the diacylglycerol substrates are not equally available for the galactosyltransferase because so many (^{14}C)diacylglycerol molecules were not galactosylated even after one hour of incubation in the presence of UDPGal.

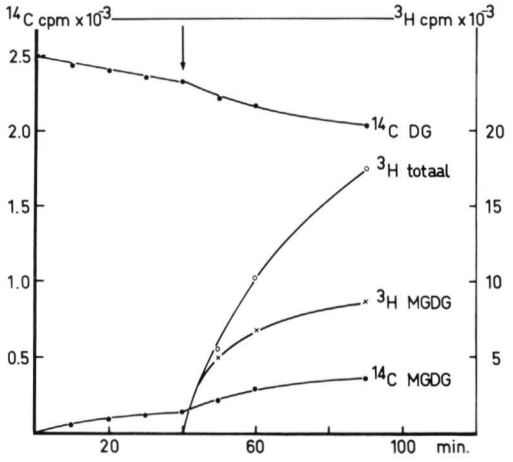


Fig. 4.7 ([^{14}C]dioleoylphosphatidylcholine was sonified with chloroplast envelopes in buffer 2. At time zero 0.8 I.U. phospholipase C was added to generate diacylglycerol (DG) in the membranes. The mixture was incubated in a shaking waterbath at 30^{0}C. At various moments aliquots were taken from the mixture and analysed. After 40 minutes UDP([^{3}H]Gal was added (arrow).

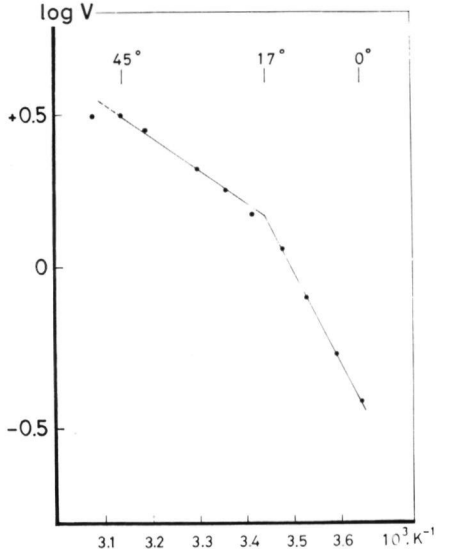


Fig. 4.8 Arrhenius plot of galactose incorporation. The logarithm of the reaction velocity is plotted on the ordinate (log µmol/mg·h). The reciprocal absolute temperature is plotted on the abscissa. Activation energies of about 21 kJ/mol above 17^{0}C and 58 kJ/mol below this temperature were calculated from the slope of the graph. For details on the incubation see 2.4.1.

The exchange of diacylglycerol moieties can be explained best by the splitting of MGDG by the galactosyltransferase into a diacylglycerol moiety and a covalently bound galactose. The diacylglycerol moiety may be released by the enzyme and exchanged with another molecule in the membrane after which the MGDG may be reassembled.

4.5 THE INFLUENCE OF TEMPERATURE ON MGDG FORMATION

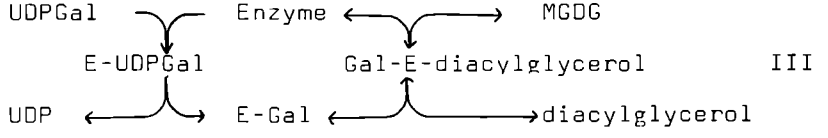
The incorporation of label from UDP(^{14}C)Gal into MGDG increased with temperature. Incorporation of label was still observed after preincubation for 5 minutes at 60°C followed by an incubation for 5 minutes in the presence of substrate at that temperature. Maximum initial rate incorporation was obtained at 50°C but 45°C was the highest temperature at which no denaturation of the enzyme became evident (fig. 4.8). When plotted according to Arrhenius the graph shows a breakpoint at 17°C commonly ascribed to a phase transition of membrane lipids (de Kruijff *et al.* 1973). However, a differential scanning calorimetric experiment showed that no lipid transition occurred in envelope or thylakoid membranes between 0°C and 60°C. Still the break in the Arrhenius plot may be caused by a phase transition of lipids. If only the lipid annulus (Mazliak 1977) of the enzyme exhibits a transition at 17°C the number of lipid molecules that change from liquidcrystalline to gel state or *vice versa* is far too small to be detected by differential scanning calorimetry. Activation energies of about 21 kJ/mol above 17°C and 58 kJ/mol below this temperature were calculated from the Arrhenius plots. Since an increase in transition temperature in aging chloroplasts has been described (Thompson *et al.* 1978), the experiment was repeated with envelopes isolated from very young and old spinach

leaves. Although the activation energy showed some variation with different batches of spinach the breakpoint in the Arrhenius plot occurred always at the same temperature, in envelopes isolated either from greenhouse grown spinach that was 5 or 12 weeks old or from spinach from the local market bought in the spring or in the fall. This might well be interpreted as a need for specific lipids in the lipid annulus of the enzyme.

4.6 DISCUSSION

Several data indicate that with isolated envelopes the galactosyltransferase activities can be studied much better than in the crude preparations used earlier. In experiments with chloroplasts, frozen after isolation, Neufield and Hall (1964) found epimerase activities to be present, as did van Hummel (1974). In addition Neufield and Hall (1964) observed the formation of DGDG with two β-glycosidic bands in 83% of the DGDG molecules instead of an α-linkage between the two galactosyl residues. Various inhibitors of galactosyl incorporation by isolated chloroplasts, such as Mn^{2+}, Fe^{3+} and Cu^{2+}, (Neufield and Hall 1964) proved to be ineffective in our experiments with isolated envelopes. On the other hand Neufield and Hall (1964) observed an almost complete inhibition of the incorporation after incubation of the chloroplast material in the presence of 50 µM UDPGal as well as 5 mM UTP which is in agreement with our experiments on the influence of various nucleotides (4.2). With respect to experiments with acetone powder preparations from spinach leaves Mudd *et al.* (1969) describe that pH optimum and incorporation ratios of galactolipids are dependent of the incubation buffer. In our experiments with envelopes at pH 7.2, however, acetate, phosphate, tris and tricine buffers resulted in identical

incorporations both in 0.1 M and 1 M concentrations. It is, therefore, likely that in crude preparations a number of changes in enzyme activity are induced indirectly.
The high specificity of the enzyme for uridine nucleotides may be advantageous as the concentration of ADPG, which is an intermediate in starch formation (Murata *et al.* 1964), may be very high in comparison to the concentration of UDPGal when chloroplasts are illuminated. On the other hand the incorporation of UDPGal may be regulated by the energy charge of the cell or chloroplast via the inhibition by UDP.
At this place it is pertinent to make a few remarks about the effects of ATP. These effects were rather difficult to explain. In some cases addition of 1 mM ATP stimulated the incorporation of galactose from UDPGal into galactolipids up to 25%, in other cases the addition of 1 mM ATP resulted in an inhibition of incorporation and of transfer of label to higher homologues. Lineweaver-Burk plots showed inhibition of incorporation at low, and enhancement at high concentrations of UDPGal, but these experiments showed poor reproducibility. In the presence of CoA, ATP was always stimulatory in comparison to reference incubations with only CoA. This relative stimulation was also observed when fatty acids were added. In view of the demonstrated presence of free fatty acids in our preparations the effect of ATP and CoA may possibly be due to activation of fatty acids and synthesis of diacylglycerol.
A simple and elegent model which can account for the competitive inhibition of UDP, the uncompetitive inhibition of oleic acid as well as the equilibrium exchange as described in 4.4 is a double displacement or ping-pong reaction which is drawn schematically:



The enzyme reacts with UDPGal forming an enzyme-galactose complex, which reacts with diacylglycerol to yield galactolipid and the

enzyme. MGDG may react with the enzyme in the absence of UDPGal yielding a diacylglycerol molecule and a covalently bound galactose. The latter may react with (another diacylglycerol molecule to form MGDG, accounting for the equilibrium exchange. The left part of scheme III appears to be irreversible. When envelopes were pulse-labelled (2.4.8) and incubated with UDP afterwards, no label was lost from the lipid phase indicating that the enzyme-galactose complex cannot react with UDP to form UDPGal. This is undoubtedly due to the higher enthalpy of UDPGal which is the driving force of the reaction, as may also be deduced from the enhancement of the incorporation of label from diacylglycerols into MGDG upon addition of UDPGal.
It was not possible to demonstrate radioactive galactose in the protein fraction after precipitation with 80% CH_3OH. Calculations from the amount of label in the proteins learned that at most one galactose could be present per 1 000 000 Dalton of total envelope protein. This does not exclude the covalent binding of galactose to the enzyme, since quite a few different proteins must be localised in the envelopes.
According to the description of the ping-pong reaction (Lehninger 1975) decrease of the second substrate causes uncompetitive inhibition and an increase of the second substrate causes an uncompetitive enhancement. Experiments with UDP(^{14}C)Gal in the presence of 100 µM diolein or phospholipase C did not alter the incorporation velocity. This phenomenon might be due to the fact that the diacylglycerol which is accessible for the enzyme is pooled and cannot be rapidly exchanged with diacylglycerol outside this pool in the membrane. This is supported by the results of the experiments with (^{14}C)diolein mentioned in 4.4. Less than 25% of the added diolein is galactosylated to MGDG even after one hour of incubation in the presence of UDPGal. The effective concentration of diacylglycerol could be diminished by addition of oleic acid whereas it was enlarged upon addition of albumin as judged by the response of the system when plotted according to Lineweaver and Burk (1934). If we take into account the low value of the K_i of oleic acid and the chemical

resemblance between oleic acid and diolein it is very likely that free fatty acids and diacylglycerols compete for the same enzymic site. The presence of fatty acids in isolated envelopes implies that values calculated from incubations in the absence of albumin should be corrected. So *in vivo* incorporation velocities might be higher, but also the K_m and the K_i of variou effectors would be higher. In the presence of 2.5 mg albumin/ml a V_{max} up to 8 µmol/h·mg protein was calculated at 30°C, if only the high affinity locus of the enzyme was regarded. The calculated activation energies also should be reconsidered, sinc these too will be influenced by the presence of fatty acids. Possibly this will lead to more reproducible values for the activation energies.

5 THE FORMATION OF DGDG, TGDG AND TeGDG

5.1 INTRODUCTION

When chloroplasts envelopes were incubated for longer periods (for example 2 h) in the presence of UDP(^{14}C)Gal at 30^{0}C, most of the label incorporated was found usually in DGDG, TGDG and TeGDG (fig. 5.1) and only a minor percentage in MGDG. According to Benson *et al.* (1958), these higher homologues are formed by linking another galactosyl moiety in a (1→6)α-way to the galactose of MGDG according to the scheme:

MGDG + UDPGal →	DGDG + UDP	II
DGDG + UDPGal →	TGDG + UDP	IV
TGDG + UDPGal →	TeGDG + UDP	V

Williams and coworkers (1976) concluded from experiments *in vivo* with *Vicia faba* that the formation of MGDG and DGDG is performed by different enzymes in a completely different way. Joyard and Douce (1976b) found different pH optima for the formation of MGDG and DGDG in experiments with isolated envelopes and concluded that there are two different enzymes. The same conclusion again was drawn by Mudd *et al.* (1969) and by Siebertz and Heinz (1977) from experiments with acetone powder extracts from spinach chloroplasts and pea seedlings respectively.
In incubations of isolated chloroplast envelopes in the presence of labelled UDPGal, we observed that the relative amounts of label in di-, tri- and tetragalactolipids were increasing with time. When the amount of label in each galactolipid was expressed

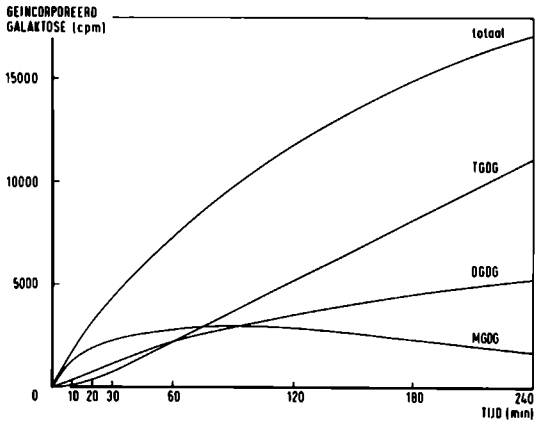


Fig. 5.1 Incorporation of label into galactolipids. In a final volume of 2.8 ml 280 µg envelope material was incubated in the presence of 0.5 mM UDPGal and buffer 2. At various moments 200 µl samples were taken from the mixture and analysed (2.3.2 and 2.3.4). The incorporated label is given for total incorporation (referred to as totaal), T(e)GDG (referred to as TGDG), DGDG and MGDG.

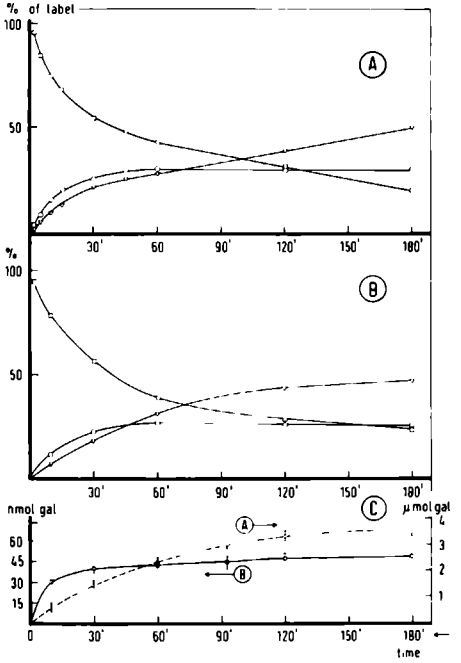


Fig. 5.2 Incorporation of label into galactolipids. The percentage of total plate cpm is given for MGDG (□), DGDG (o) and T(e)GDG (▽). A) similar to fig. 5.1 but in the presence of 2 mM UDPGal. B) Idem but in the presence of 5 µM UDPGal. C) Absolute incorporation of radioactivity per mg protein refferring to A and B.

as the percentage of total label incorporated up to that moment, a new aspect of the incorporation could be seen (fig. 5.2). Since extrapolation to time zero leads to 100% MGDG, it is likely that the first reaction product is MGDG which is lateron galactosylated to DGDG. This would imply that the reactions I and II are coupled and, hence, that the enzyme must be able to distinguish the newly synthesized MGDG from the bulk MGDG in the membrane. This might occur in either of two manners: a) the 2 MGDG species differ in fatty acid composition and the galactosyltransferase catalysing DGDG formation prefers some distinct fatty acids with high specificity and/or b) the enzymes are linked spatially, *i.e.* the products of the first reaction constitute a separate pool from which the next enzyme chooses its substrates. At the start of the experiment this pool must be supposed to be empty in view of the lag time in the labelling of DGDG and higher homologues. Support for hypothesis a) was given by experiments of Mudd *et al.* (1969) in which acetone powder precipitates from spinach leaves were supplied with UDP(^{14}C)Gal and various diacylglycerols. The incorporation of label into galactolipids was found to depend on the degree of unsaturation of the fatty acyl moieties of the diacylglycerols. Similar effects on the formation of DGDG were reported by Siebertz and Heinz (1977) who used acetone precipitates and Triton X-100 extracts from pea seedlings supplied with UDP(^{14}C)Gal and various MGDG species. Both papers report increased incorporation for the more unsaturated lipid substrates. On the other hand, in many species DGDG differs from MGDG in fatty acid composition and in these cases DGDG is characterised by a higher percentage of saturated fatty acids (Jamieson and Reid 1971, Heinz 1977).

In fig. 4.2 it was shown that the incorporation of label into galactolipids depends strongly on the concentration of UDPGal. However, when we look at the percentage of label in each galactolipid, this appears to depend only on the incubation time and not on UDPGal concentration as can be seen from an example in fig. 5.2. In this figure 2 incubations are shown with substrate

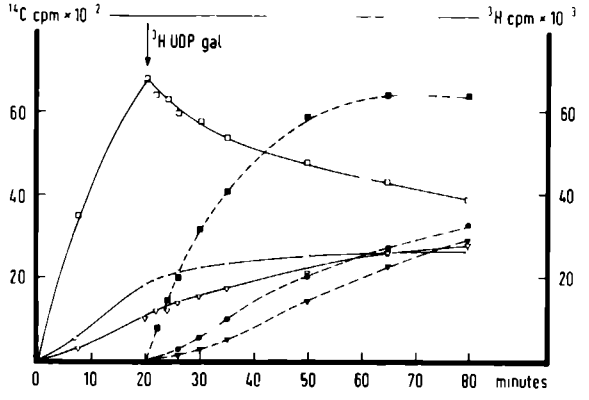


Fig. 5.3 Incorporation of galactose in a double labelling experiment. The drawn-out lines represent the amount of ^{14}C-labelled galactose in MGDG (□), DGDG (o) and T(e)GDG (▽). After removal of UDP(^{14}C)Gal (2.4.8), UDP(^{3}H)Gal was added and incorporated into MGDG (■), DGDG (●) and T(e)GDG (▼), as shown by the dashed lines.

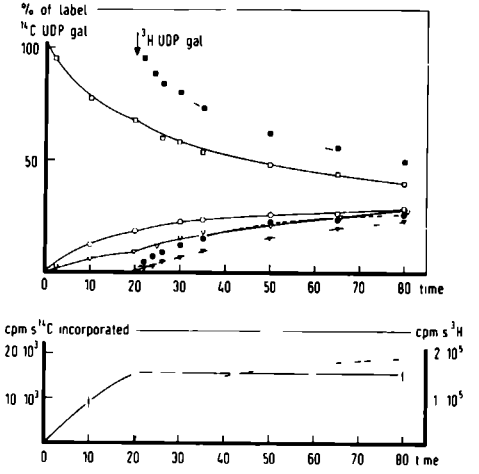


Fig. 5.4 Incorporation of galactose in a double labelling experiment. Percentages of total plate cpm were calculated for both ^{14}C-label (drawn lines) and ^{3}H-label (dashed lines). The ^{14}-label in MGDG (□), DGDG (o) and T(e)GDG (▽) is further indicated with open dots. Total incorporation is given separately and shows that total ^{14}C radioactivity remains constant after 20 minutes, when UDP(^{14}C)Gal was replaced by UDP(^{3}H)Gal (2.4.8).

concentrations differing by a factor 400; yet the percentage of label in each galactolipid is the same in both incubations. This makes it plausible that the reactions are strongly linked, supporting hypothesis b).
This conclusion was strengthened by experiments designed to test the influence of various effectors, that were succesfully used in the previous chapter, on the labelling patterns. Both UDP and oleic acid proved to be completely ineffective, that is to say, the amount of label incorporated was diminished, but the percentages of each galactolipid at any moment were identical to those of control experiments in the absence of the effectors. Although the conclusion that the reactions are linked, was firm now, we still were unable to fit the various findings into reaction scheme II, IV and V.
The only effector that influenced the labelling rates of the galactolipids was albumin (fig. 4.5). However, the influence was rather small and occurred at high concentrations (5 mg albumin/ml). This suggests that this effect of albumin is not very specific and might be due to a general effect on the membrane.

5.2 PULSE LABELLING EXPERIMENTS

To test the validity of hypothesis b), we decided to perform a double-labelling experiment. Envelope membranes were incubated for 20 minutes at 30°C in the presence of UDP(^{14}C)Gal. After removal of the substrate (2.4.8), the envelopes were resuspended in buffer 2 by light sonification and incubated at 30°C in the presence of UDP(^{3}H)Gal. At various moments aliquots were taken from the reaction mixture and analysed (2.3.2 en 2.3.4). Fig. 5.3 shows the results of such an experiment, the incorporated label

being plotted versus time, and in fig. 5.4 the same results are plotted in percentage of total incorporation. From the shift in ^{14}C-label it is clear that the transfer of ^{14}C-label from MGDG into DGDG and higher homologues proceeds as if the incubation had not been interrupted. The ^{3}H-label, however, is incorporated with exactly the same distribution pattern as was observed for the ^{14}C-label in the first incubation. Both hypotheses a) and b) presented above seem inadequate to explain these data, since in both cases one would expect that immediate formation of ^{3}H-labelled DGDG were possible. Furthermore a pulse-labelling experiment was performed in which UDPGal was omitted in the second incubation. The transfer of ^{14}C-label from MGDG and DGDG and higher homologues proceeded at the exactly same relative rates in the presence as in the absence of UDPGal. The most ready explanation for this finding was, that the assumed galactosylation of MGDG and DGDG did not take place at all in these preparations. This forced us to reject both hypotheses a) and b), and the reaction mechanism described in II, IV and V. Apparently, DGDG can be synthesised only from newly formed MGDG, irrespective of the amount of MGDG already present, and its formation does not require UDPGal.

The same reasoning also applied for the formation of TGDG and TeGDG. One model meeting these requirements was that MGDG is the galactosyldonor for the formation of DGDG and higher homologues. So we suggest the following scheme for the formation of DGDG:

UDPGal + diacylglycerol	→	MGDG + UDP		I
2 MGDG	→	DGDG + diacylglycerol		VI

For the formation of T(e)GDG one may conceive analoguously:

MGDG + DGDG	→	TGDG + diacylglycerol		VII
MGDG + TGDG	→	TeGDG + diacylglycerol		VIII

This implies the possibility that one galactosyltransferase is responsible for all these syntheses assuming a low enzymic specificity for the carbohydrate moiety of the galactose accepting lipid.

Afterwards we realised that the UDPGal independent galactosyl-transfer was already demonstrated in fig. 5.2, since in the

incubation in the presence of 5 µM UDPGal after 30 minutes the UDPGal was exhausted, whilst intergalactolipid transfer of label still continued.

5.3 INFLUENCE OF TEMPERATURE ON SYNTHESIS OF HIGHER HOMOLOGUES

In some of the pulse labelling experiments of the previous section, we used a time consuming ultrecentrifugation procedure to separate the envelopes from the substrate. To be certain that this procedure had not a significant influence on the distribution of label, for example because of differences in centrifugation time, we did the following experiment. A certain amount of envelope material was resuspended in 1 ml buffer 2 and 150 µl 2 mM UDP(^{14}C)Gal was added. After an incubation of 25 minutes at 30^{0}C, 3 ml icecold buffer was added and the mixture was centrifuged. The supernatant was discarded and the pellet was resuspended in 1.32 ml buffer 2. From this mixture three portions of 0.2 ml were taken (samples 1, 2 and 3). To samples 1 and 2 25 µl 2 mM UDP(^{3}H)Gal was added (at 0^{0}C). Methanol (1.5 ml) was added immediately to sample 1, whilst samples 2 and 3 were incubated for 30 minutes at 30^{0}C respectively in the presence and in the absence of UDPGal. The centrifugation procedure was repeated twice with the remaining envelope suspension. The pellet was now resuspended in 660 µl buffer 2 resulting in samples 4, 5 and 6 (each 200 µl). These were incubated exactly the same as samples 1, 2 and 3 respectively. The percentage label in each galactolipid was calculated and is shown in table 5.1.

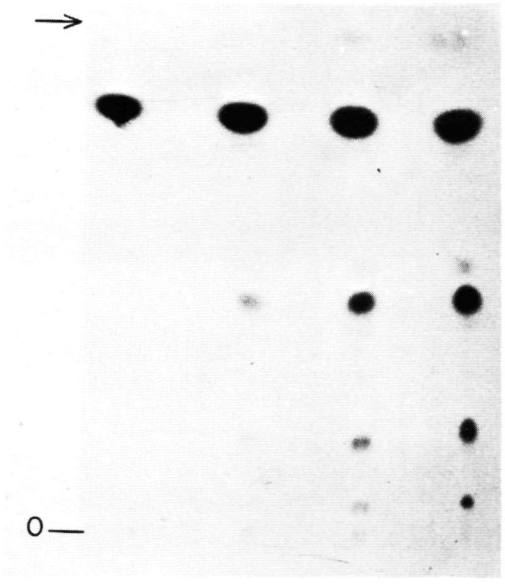


Fig. 5.5 The influence of the temperature on the formation of MGDG, DGDG and T(e)GDG. From left to right the incorporated ^{14}C-label is shown for incubations at 0^0C, 15^0C, 30^0C and 45^0C. The *Rf* values of MGDG, DGDG, TGDG and TeGDG were respectively: 0.76; 0.44; 0.20 and 0.08. Origin and front are indicated respectively with O and →.
All samples were incubated in the presence of 0.5 mM UDPGal and buffer 2.

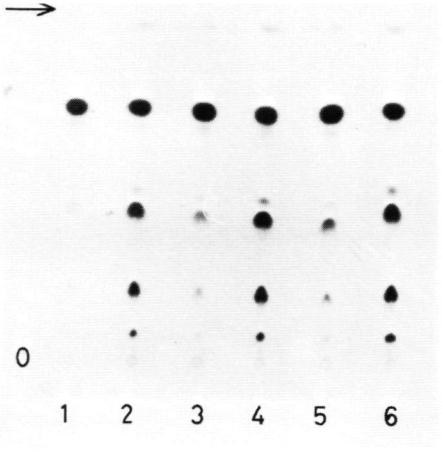


Fig. 5.6 The influence of pH on the formation of galactolipids. Samples 1, 3 and 5 were incubated at pH 8.5 at 30^0C for respectively 30, 60 and 90 minutes. Samples 2, 4 and 6 were incubated similarly but at pH 7.2. *Rf* values and indications are the same as in fig. 5.5. Total incorporation is given in table 5.3. From samples 5 and 6 the activities were measured (2.3.4).

	5	6
MGDG	86%	48%
DGDG	9%	26%
TGDG	3%	20%
TeGDG	2%	6%

Table 5.1 Distribution of label in envelope lipids after various treatments
A: number of centrifugations B: minutes of incubation at 30°C

sample	A	B	MGDG		DGDG		T(e)GDG		total cpm	
			^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
1	1	0	100	66	0	21	0	10	500	3100
2	1	30	64	49	21	27	14	21	8000	3000
3	1	30	-	47	-	27	-	23	-	3100
4	3	0	100	65	0	21	0	11	450	3000
5	3	30	68	48	18	27	15	20	8200	3050
6	3	30	-	48	-	27	-	22	-	3100

As can be seen from the table, the labelling patterns are not altered by the centrifugation procedure. It appears that at low temperature the interlipid galactosyltransferase does not operate. In the experiments mentioned above, sample 4 is kept -at 4°C- at least 1.5 h longer than sample 1, yet the ^{14}C-labelling pattern is identical. Similarly it appears that the galactosyltransferase activity does not decrease upon centrifugation of the envelopes.
The next step was to investigate at which temperature the inactivation of the interlipid galactosyltransferase occurred. For this purpose envelopes were incubated for 60 minutes in the presence of UDP(^{14}C)Gal at various temperatures. The results are shown in table 5.2.

Table 5.2 The temperature dependence of interlipid galactosyltransferase

temperature	% label in: MGDG	DGDG	T(e)GDG	total cpm
10°C	92.5	4	3.5	3300
17°C	92	4.5	3.5	4500
20°C	90	6.5	5	4800
30°C	79	13	8	6200
40°C	69	18.5	12.5	6700

From the table it appears that the percentage of label in DGDG and T(e)GDG increases as the temperature rises above 17°C, which, incidentally, is equal to the temperature at which the break point occurs in the Arrhenius plot for the UDPGal-diacylglycerol galactosyltransferase (4.5).
In other experiments it was shown that in some cases the activity of the interlipid galactosyltransferase decreased even further upon cooling the incubation mixture to 0°C (fig. 5.5). At this temperature 100% of the incorporated label was found in MGDG, whilst other galactolipids remained unlabelled.
The highest activity of interlipid galactosyltransferase was observed at 45°C, which is also the maximum temperature for sustained UDPGal-diacylglycerol galactosyltransferase. Although interlipid galactosyltransferase activities were rather variable in different batches of spinach -compare fig. 5.2 and table 5.2- the temperature of 17°C and 45°C, at which changes occur, are remarkably constant. Moreover both galactosyltransferases exhibit changes in activities at these 2 temperatures. It is tempting therefore to attribute these similarities to a spatial relationship of the enzymes in the envelope. According to Douce and Joyard (1978) these enzymes operate in a multi-enzyme sequence together with desaturases and other enzymes involved in galactolipid biosynthesis. If this were true both enzymes may well possess similar structural lipids or share a common lipid annulus.

5.4 THE INFLUENCE OF pH ON THE SYNTHESIS OF DGDG AND T(e)GDG

When the pH of a buffer in which envelope material is suspended is brought below pH 7.0, an enzyme is activated (Heinz 1973) in the membranes, which catalyses transfer of acylgroups, preferably

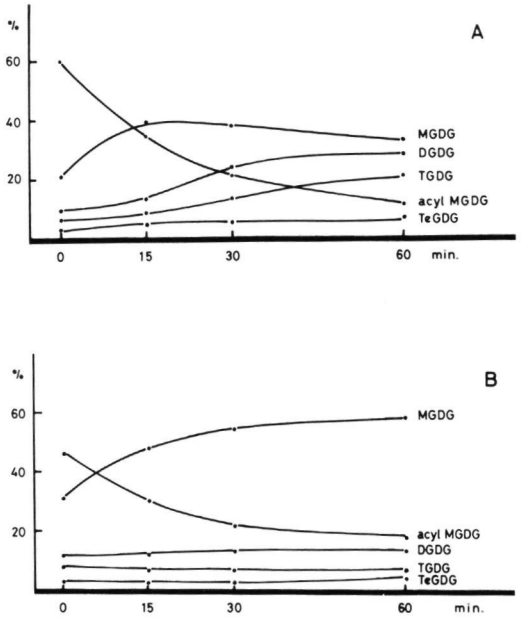


Fig. 5.7 Interlipid galactosyltransferase activity at pH 7.2 (A) and pH 8.5 (B). The percentage of label in each component is plotted on the ordinate and on the abscissa the time of incubation is given. Membranes were pulse-labelled at pH 6 for 20 minutes. After removal of excess UDPGal by centrifugation (2.4.8), samples were incubated in 1 M tricine/KOH buffer pH 7.2 or in 1 M tris/HCl buffer pH 8.5 in the presence of 10 mM $MgCl_2$. The incubation temperature was 30°C.

from DGDG, to the primary hydroxylgroup of the galactosylmoiety of MGDG (Heinz *et al.* 1978a). Because both MGDG and DGDG are consumed in this reaction, the incorporation of label from UDP(^{14}C)Gal into DGDG and T(e)GDG may be less than in incubations at pH 7.2. It is, however, not easy to decide if the enzymic activities are decreased.

Also at pH 8.5 we observed a decreased incorporation of label into DGDG and T(e)GDG. The total incorporation was inhibited for only 20% (table 5.3) but the incorporation into DGDG and T(e)GDG was inhibited for more than 70% (fig. 5.6), resulting in even a higher amount of label in MGDG at pH 8.5 than at pH 7.2.

Table 5.3 Incorporation of label from UDP(^{14}C)Gal at pH 7.2 and pH 8.5

incubation time:	2'	10'	20'	60'	90'	120'
cpm/µg protein pH 7.2	4.7	19.0	30.6	56.4	66.0	71.1
cpm/µg protein pH 8.5	2.9	14.6	24.6	44.2	52.6	59.0

To get a deeper insight in the effects of high pH on the interlipid galactosyltransferase activity we also investigated the effect of high pH in the absence of UDPGal. Envelope membranes were pulse-labelled at pH 9.0 at 30°C to get a high percentage label into MGDG. After 30 minutes the mixture was cooled, the UDP(^{14}C)Gal washed away (2.4.8) and the envelopes were incubated at several pH for various times. The results are shown in table 5.4.

Table 5.4 The effect of pH on interlipid galactosyltransfer in the absence of UDPGal, after preincubation at pH 9.0

incubation:	%MGDG	%DGDG	%T(e)GDG	total cpm
0' at pH 7.2	80	15	5	6200 ± 400
5' at pH 7.2	69	21	10	6000 ± 400
30' at pH 7.2	55	28	17	6300 ± 400
60' at pH 7.2	46	30	25	6100 ± 400
60' at pH 8.0	73	19	8	5900 ± 400
60' at pH 8.5	72	19	9	6100 ± 400
60' at pH 9.0	73	19	8	6000 ± 400

Table 5.4 and fig. 5.6 show that the interlipid galactosyltransferase is inhibited at high pH, both in the presence of UDPGal and in its absence. After preincubation at pH 9.0 the enzyme can be reactivated by incubation at pH 7.2, but hardly at pH 8 or higher. As can be seen from table 5.4, the amount of label transferred in 60 minutes at pH 8 was about equal to the amount transferred in three minutes at pH 7.2.
In other experiments envelopes were pulse-labelled at pH 6 and subsequently incubated at pH 7.2 and pH 8.5. Fig. 5.7 shows that also in this case transfer of label into DGDG and T(e)GDG was only measurable at pH 7.2.
Attention must be given to another phenomenon apparent from fig. 5.7. After pulse-labelling for 20 minutes at pH 6 acylMGDG accounts for the highest percentage of label in galactolipids. Incubation of these samples at higher pH always resulted in a

rapid decrease of label in acylMGDG and its reappearance in the more conventional galactolipids. At pH 8.5 the conversion of acylMGDG into MGDG was even the only event that was observed, since the interlipid galactosyltransferase was not active in these conditions. At pH 7.2 the simultaneous activity of the acylMGDG hydrolysing enzyme and the interlipid galactosyltransferase results in an initial increase in MGDG label, followed by a decrease. Hence, acylMGDG can be considered to be a precursor of DGDG and T(e)GDG under these circumstances. We have not ascertained whether the acyl moiety from acylMGDG is released into the medium, or is transferred to another unlabelled lipid compound. Hence, we do not know whether the enzyme is a hydrolase or an acyltransferase.

5.5 DISCUSSION

In the previous sections we have reported various features of galactolipid formation which provide evidence for a UDPGal independent galactosyltransfer according to:

2 MGDG → DGDG + diacylglycerol VI

MGDG was always the first compound to be labelled, since the percentage of label incorporated in this fraction always extrapolates to 100% at time zero. So it seems clear that MGDG is a necessary precursor to DGDG and higher homologues. This is supported by the fact that the curves for the appearance of label in DGDG and higher homologues are sigmoidal, with very low rates of incorporation after short incubations.
It was further observed that the relative rates of labelling were very similar under the following conditions: presence of excess UDPGal, exhaustion of UDPGal, the addition of oleic acid or UDP, replacement of ^{14}C-label by ^{3}H-label and the complete

absence of UDPGal after pulse-labelling.
It may be remarked that the reaction according to VI does not seem to involve a water-soluble intermediate, since no label was lost from the lipid phase in these experiments. Furthermore results of former research regarding the biosynthesis of galactolipids do not seem to be incompatible with the hypothesis outlined above and in some cases they can be considered as a support for it. In the experiments of Ongun and Mudd (1968), where the enzymes first were freed of lipids, the best rate of incorporation from UDP(^{14}C)Gal was obtained when galactolipids were added. When this lipid fraction, containing both MGDG and DGDG, was incubated with the protein extract, most of the label was found in MGDG, which cannot be understood unless the MGDG added was first transformed into diacylglycerol. These authors also report experiments designed "to wash out the label from MGDG to DGDG by incubating the chloroplasts with excess of cold UDPGal after they had incorporated all the radioactivity from 2 nmol UDP(^{14}C)Gal into lipids". A 2 h chase resulted in a decrease of the ratio of radioactivity in MGDG/DGDG from 2.22 to 1.27. They further report: "Although to a much lesser extent, there was also some drop in this ratio without any addition of cold UDPGal after the first hour of incubation". Our experiments in which a more purified enzyme system was used differ in a quantitative way from such results but it is clear, a posteriori, that a reaction as indicated in VI was occurring also in the crude enzyme preparations.
Other pathways of DGDG formation can be excluded for isolated envelopes from spinach chloroplasts, although several authors report alternative pathways in experiments with different material. Blee and Schanz (1978a) in agreement with work by Lin and Chang (1971) report that after incubation of fractions from *Euglena gracilis* in the presence of UDP(^{14}C)Gal, the ratio of labelled MGDG/DGDG is independent of time. This leads to the conclusion that probably both galactolipids are formed from UDPGal. However, the incorporation velocities were quite low on a protein basis (about 0.1% of our value) and only a modest

percentage of the UDPGal offered was incorporated (compare fig. 5.2b where all UDPGal was incorporated). So future research will be necessary in order to establish whether different mechanisms for biosynthesis occur in *Euglena* and spinach or whether the present discrepancies can be reconciled.

Siebertz and Heinz (1977) studied the formation of DGDG from MGDG and UDPGal in cell free preparations from pea seedlings. Under optimal conditions they were able to synthesise pure DGDG from UDPGal and MGDG offered to acetone precipitates or Triton X-100 extracts. Highest activity was found in the 1 500 000 m/s^2 supernatant prepared from homogenates of young seedlings. This seems to indicate that the enzyme is a soluble protein of the cytoplasm, although its presence in the supernatant might be due to the fragility of the plastid envelope. The latter is even more likely, since in the experimental conditions 3% Triton X-100 is used to dissolve the enzyme preparation, which one would not expect to be necessary for a soluble enzyme. In these experiments the incorporation velocities are only 0.1% of those established in isolated chloroplast envelopes (Joyard and Douce 1976a) (4.1). Furthermore it was observed by Heinz *et al.* (1978a) that 0.9% Triton X-100 strongly inhibits the formation of DGDG in isolated envelopes. This makes the incorporation of the results of Siebertz and Heinz (1977) in terms of which pathways are used *in vivo* in DGDG formation very precarious.

Attention should be paid to the surprisingly high rates of labelling of T(e)GDG, observed in the purified envelope system. In older experiments, where chloroplasts or acetone powders were used, the ratios of cpm in MGDG/DGDG usually were very high (Mudd *et al.* 1969, van Hummel *et al.* 1975), but when DGDG synthesis was significant, also some radioactivity was seen in T(e)GDG (Ongun and Mudd 1968, Webster and Chang 1969). However, the occurrence of these compounds *in vivo* is rare and concentrations were always low in comparison with MGDG and DGDG (Poincelot 1973, Leese and Leech 1976). Their formation might be thought to proceed by the same galactosyltransferase as assumed for the formation of DGDG according to the scheme (5.2):

MGDG + DGDG → TGDG + diacylglycerol VII

A possible alternative for this scheme could be:

2 DGDG → TGDG + MGDG IX

In our view, however, this scheme is not very probable. The galactosyltransfer between 2 MGDG molecules requires the change of the β-galactosyl linkage in MGDG into an α-bond, common for intergalactosyl linkages in DGDG and TGDG. A change in configuration occurs also in the formation of MGDG according to I. However, reaction IX proceeds without change in configuration. In view of the rare event of synthesis of TGDG and the good evidence for scheme VI, it seems simpler to assume VII than to postulate the entirely different mechanism shown in IX.

The observation (5.4) that interlipid galactosyltransfer was inhibited above pH 7.5 both in the presence and in the absence of UDPGal offered us the opportunity to see if envelopes isolated under these conditions have a different composition from those isolated at pH 7.2. In other words to see if the amounts of diacylglycerol, shown to be present by Joyard and Douce (1976a) are present *in vivo* or perhaps are the result of the activity of the interlipid galactosyltransferase during the isolation procedure.

6.1 INTRODUCTION

Rather diverse results have been published from analyses of the lipid composition of chloroplast envelopes. A summary of the data for some common lipids is presented in chronological order in table 6.1.

Table 6.1 The composition of isolated envelopes. Data are reported on the quantities (in percentages by weight) of some acyllipids of major significance.

Source: spinach	MGDG	DGDG	SL	PC	PG	mg lipid/ mg protein
Poincelot (1973)	33	34	1	9	3	1.0
Douce *et al.* (1973)	22	32	5	27	8	1.2
Hashimoto and Murakami (1975)	8.5	29	5.5	27.5	13	-
Joyard and Douce (1976)	25	32	6	20	8	1.2
Poincelot (1976b)	27	33	0.1	25	6	1.0
Vicia faba						
Mackender and Leech (1974)	29	32	-	30	9	-
Compare:						
Spinach lamellae (Douce 1973)	35	18	5	3	7	0.4

The ratio of MGDG over DGDG in table 6.1 varies between 0.3 and 1.0. Such variations were also reported by Joyard and Douce (1976b) and seemed to depend on the batch of spinach and the isolation procedure. By measuring the amount of galactose in

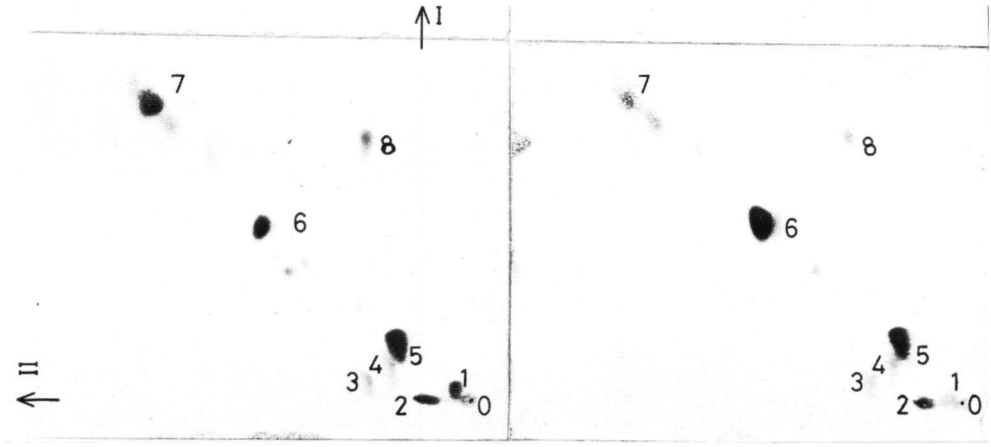


Fig. 6.1 Two dimensional thin layer chromatograms of envelope lipids. Envelopes, equivalent to 0.2 mg protein isolated at pH 7.2 (left, with arrows for indication of elution) and pH 8.5 (right), were extracted (2.3.2) and chromatographed (2.5.1). Spots were visualised by heating after spraying with 25% H_2SO_4 in CH_3OH. *0*=origin, *1*=TGDG, *2*=PC, *3*=PG, *4*=SL, *5*=DGDG, *6*=MGDG, *7*=diacylglycerol (amidst carotenoids) and *8*= free fatty acids.

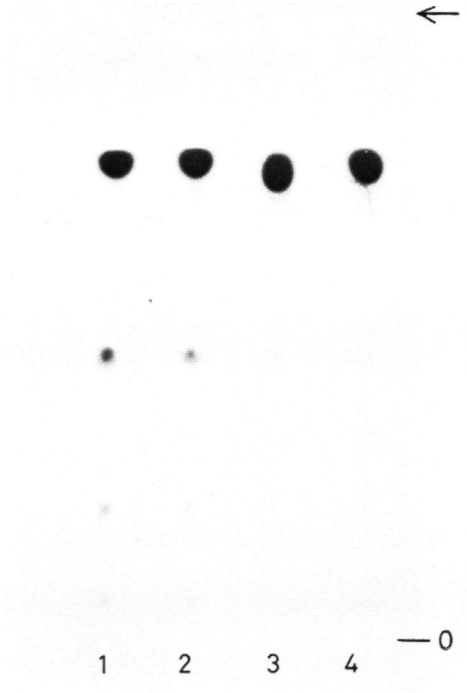


Fig. 6.2 Galactosyltransferase activities after various treatments. Envelope material (±50 µg) was incubated for 60 minutes at 30°C in the presence of 0.2 mM UDP(^{14}C)Gal. *1* Envelopes isolated at pH 7.2 incubated at pH 7.2; *2* idem *1* but incubated at pH 8.5; *3* envelopes isolated at pH 8.5 incubated at pH 7.2; *4* idem *3* but incubated at pH 8.5. Origin and front are indicated by respectively 0 and arrow.

MGDG and DGDG after separation by thin layer chromatography, we obtained similar results. Envelopes incubated for 30 minutes at 30°C in buffer 2 at pH 7.2 showed a MGDG/DGDG ratio of 0.3 whereas envelopes incubated similarly but in the presence of 2 mM UDPGal contained roughly equal amounts (on a molar basis) of mono- and digalactolipids. This is in marked contrast to the ratio in lamellae which is about 2.
Only Joyard and Douce (1976a,b) report large amounts of diacylglycerol in spinach chloroplast envelopes, which may amount up to 15% of dry lipid weight. In our experiments too, diacylglycerol was found to be a major membrane lipid (fig.6.1). However, as we shall see lateron, its presence depends on the interlipid galactosyltransferase activity during isolation procedure.

6.2 COMPARISON OF ENZYMIC ACTIVITIES OF ENVELOPES ISOLATED AT pH 7.2 AND pH 8.5

Chloroplast envelopes were isolated from spinach leaves according to the method of Joyard and Douce (1976b) (2.2.2). From the same batch of spinach, envelopes were isolated identically with one exception: in all solutions the 0.1 M tricine/KOH buffer pH 7.2 was replaced by a 0.1 M tris/HCl buffer pH 8.5. At the end of the procedure we had two batches of envelopes, both chlorophyll-free and in both cases the yield was about 1.5 mg/kg spinach leaves. Both preparations incorporated label from UDP(^{14}C)Gal into various galactolipids but not to the same extent. The interlipid galactosyltransferase was markedly inhibited in the envelopes isolated at pH 8.5 and in an incubation at pH 8.5 all label was incorporated into MGDG irrespective of the time of incubation (fig. 6.2). This inhibition seemed to be irreversible. In contrast, the incorporation of galactose into DGDG and T(e)GDG

in envelope isolated at pH 7.2 was only partly inhibited at pH 8.5 (5.4). At low pH acylMGDG was labelled in both preparations and this lipid was also degraded to MGDG by both preparations at pH 7.2 as well as at pH 8.5.
In conclusion we can say that apparently the interlipid galactosyltransferase is inactivated when envelopes are isolated in the absence of UDPGal at pH 8.5. Apart from the interlipid galactosyltransferase the envelopes seemed to be fully intact when isolated at pH 8.5.

6.3 THE LIPID COMPOSITION OF ENVELOPES ISOLATED AT pH 7.2 and pH 8.5.

In preliminary experiments we made lipid extracts from small but equal amounts of envelope material isolated at pH 7.2 and pH 8.5. These extracts were chromatographed in two directions (2.5.1). Subsequently these chromatograms were sprayed with 25% H_2SO_4 in CH_3OH and immediately after heating photographs were taken (fig. 6.1). One can clearly see the differences in MGDG, DGDG, TGDG and diacylglycerol content. Although quantitative evaluation was not the purpose of this experiment, it is obvious that in the sample isolated at pH 8.5 the MGDG/DGDG ratio is higher than unity, whereas in the sample isolated at pH 7.2 it is lower than unity. A large difference can also be seen for diacylglycerol and TGDG. These are only significant components when the membranes are isolated at pH 7.2. We therefore decided to analyse envelope membranes at pH 8.5 gaschromatographically in order to see if these qualitative differences could be confirmed by a quantitative analysis. The results are shown in table 6.2.

Table 6.2 The acyllipid composition of envelopes isolated at pH 8.5

	MGDG	DGDG	PC	PG	SQ	DG	TGDG
µg/mg protein	570	455	180	120	34.5	22.4	23
%	36	29	17	7.6	2.2	1.4	1.4

This is in good agreement with the qualitative results of fig. 6.1. The ratio MGDG/DGDG is 1.25 by weight and 1.14 on a molar basis. This is in contrast to the data of table 6.1 where this ratio was found to oscillate between 0.3 and 1. At the same time the amounts of TGDG and diacylglycerol were very much lower than those reported by Joyard and Douce (1976a,b). These authors find 60 µg TGDG and 10 µg TeGDG and up to 300 µg diacylglycerol per mg protein. The total amount of galactolipids and diacylglycerol reported by Joyard and Douce (1976b) is 950 µg/mg protein and agrees reasonably well with the 1.07 mg/mg protein observed by us.

Joyard and Douce (1976a) report that the main constituent fatty acids in diacylglycerol are C_{16-3} and C_{18-3}. In contrast, we have found in envelopes isolated at pH 8.5 that the major fatty acid species of diacylglycerol are C_{14-0} (28%), C_{16-0} (10%), C_{16-2} (14%), C_{18-1} (13%), C_{18-2} (10%) and C_{18-3} (15%), whereas C_{16-3} occurs only in trace amounts. In view of these results it is well possible that the diacylglycerol observed by Joyard and Douce (1976a,b) is generated from MGDG by the action of the interlipid galactosyltransferase.

6.4 DISCUSSION

The composition of isolated chloroplast envelopes is characterised by a low ratio (0.3-1) of MGDG. Since the experiments mentioned above show that considerable variations in the ratio MGDG/DGDG

and large differences in diacylglycerol content can be experimentally induced, it seems quite logical to assume that the analytical data reported so far (table 6.1) may be different from the situation *in vivo*. We showed that the interlipid galactosyltransferase is inhibited at pH 8.5. Therefore we believe that the composition of envelopes isolated at pH 8.5 reflect the lipid composition *in vivo* better than the composition of envelopes isolated at pH 7.2. In our opinion these present data are further evidence for the existence of a UDPGal independent interlipid galactosyltransferase. The activity of this transferase during the isolation seems remarkable, since this enzyme has very low activity at low temperature (5.3). However, the length of the isolation procedure (5 to 6 hours) must be considered as a significant factor.
An interesting remark can be made about the fatty acid composition of diacylglycerol present in the envelopes isolated at pH 8.5. It is very well possible that this compound is (partly) generated by action of a phospholipase C on envelope lecithin, which contains mainly C_{16-0}, C_{18-1}, C_{18-2} and C_{18-3} fatty acids.

7 GENERAL DISCUSSION

In chapter 4 we confirmed scheme I, firstly proposed by Benson *et al.* (1958). In view of these results and those of other authors we may safely assume that the formation of MGDG *in vivo* also occurs by galactosylation of diacylglycerol in the chloroplast envelope.

As was already stated in chapter 5 and 6, all results from experiments with isolated envelopes regarding the formation of DGDG and quite probably also the higher homologues can be explained by the action of the interlipid galactosyltransferase shown in schemes VI, VII and VIII. However, the question may be raised whether the information on the interlipid galactosyltransferase is sufficient to explain the known facts regarding the biosynthesis of galactolipids *in vivo*.

Some of the most important facts are:

a) T(e)GDG synthesis may be quite active in chloroplast envelopes but occurs only rarely *in vivo*.

b) In many species of plants MGDG and DGDG are characterised by a different fatty acid composition.

A major difference with galactolipid formation *in vivo* is that the mechanism which regulates the transport of newly formed galactolipids from the envelope to the thylakoids does not function in isolated envelopes. Hence, in these envelopes MGDG will be formed by galactosylation of the diacylglycerol generated by reactions VI, VII and VIII. The activity of the interlipid galactosyltransferase will result in the accumulation of galactosyl moieties on those lipids that are able to accept these, resulting in unphysiologically high concentrations of DGDG and T(e)GDG. The formation of TGDG *in vivo* is rare, and

restricted to older leaves. It may be supposed that in these senescent leaves thylakoids are being degraded and no longer constitute the sink for the galactolipids synthesized in the envelopes. This senescence, therefore, may have similar effects as induced by swelling of isolated chloroplasts: the interlipid galactosyltransferase in the envelopes is no longer restricted and, in the absence of UDPGal, the MGDG decreases and simultaneously DGDG, TGDG and diacylglycerol increase.
The affinity of the various galactolipids for the transferase seems to decrease with increasing numbers of galactosyl residues in the galactolipid. TeGDG which has detergent properties is apparently not fit to accept another galactosyl moiety. The low content of TeGDG in envelope membranes indicates that also TGDG is a poor substrate.
The differences in fatty acid composition between MGDG and DGDG are well known (Jamieson and Reid 1971, Heinz 1977). The major fatty acids in MGDG (in spinach) are C_{18-3} and C_{16-3}, whereas DGDG contains mostly C_{18-3} and C_{16-0}. One may suppose, therefore, that DGDG is formed (according to VI) from MGDG containing C_{18-3} and C_{16-0} fatty acids or that it is formed from a common MGDG followed by an exchange of the C_{16-3} acyl moiety against palmitic acid.
In chloroplast envelopes isolated according to Joyard and Douce (1976b), Heinz *et al.* (1978b) observed that upon addition of UDP(^{14}C)Gal labelled DGDG was formed containing almost exclusively C_{18-3} and C_{16-3} fatty acyl moieties. If this is true, it means that in isolated envelopes the specificity for MGDG containing C_{16-0} fatty acids is lost or that the acyltransferase is inactivated. Since Heinz *et al.* (1978b) also reported that the galactosyl moieties were labelled to the same extent, it appears that the interlipid galactosyltransferase synthesises DGDG preferably from newly formed MGDG, without discrimination between donor and acceptor MGDG. This might be due to the very high rate of MGDG formation (an isolated envelope preparation would double its lipid mass in 30 minutes if enough diacylglycerol were supplied), but also the unnaturally large amounts of diacyl-

glycerol, generated during isolation, may be a significant difference with the situation *in vivo*.
If we follow the hypothesis outlined above, *i.e.* that the presence of TGDG indicates a block in the transfer from the envelopes to the thylakoids, we would expect that chloroplasts containing significant amounts of TGDG should also have some DGDG with C_{16-3}, albeit in rather lower percentage due to the preponderance of common DGDG from the thylakoids.
Williams *et al.* (1975a) fed *Vicia faba* leaf discs $^{14}CO_2$ during 10 minutes. After a short period the outer galactose of DGDG was labelled to the same extent as the galactosyl moiety of MGDG. This is easily explained by a rapid exchange between these molecules according to VI. The innermost galactosyl moiety of DGDG, *i.e.* the one linked to the glycerol was labelled quite slowly, reaching the same specific activity as the other galactosyl moieties only after 48 hours in mature leaves. This may be considered an argument in favor of separate pools of MGDG, one constituting the galactosyl donor and regenerating diacylglycerol and another pool of acceptor MGDG, *e.g.* characterised by a different fatty acid composition. In view of the recent data by Heinz *et al.* (1978b) such a separation of pools may not be observable in experiments with isolated envelopes. This may be understandable, since some enzymic parameters, such as sensitivity to low temperature, have also changed after the isolation. Moreover, the generation of diacylglycerol during the isolation procedure may flood both donor and acceptor pools causing a decreased enzymic specificity. Therefore such pools may very well exist *in vivo*.
It must be concluded that the present data are not sufficient for a full explanation of galactolipid formation *in vivo*. Additional evidence regarding the occurrence of donor and acceptor pools and the possibilities of a) acyltransfer from and to galactolipids, b) desaturation of galactolipid acyl moieties, and c) diacylglycerol generation by hydrolysis of phosphatidylcholine or phosphatidic acid, will be required to obtain a more complete picture. Nevertheless, the difference in enzymic

mechanism for the synthesis of MGDG and DGDG, demonstrated in this thesis and the differences in their dependence on important endogenous and environmental factors may contribute substantially to a further understanding of the ways in which the composition of the major thylakoid lipids is regulated.

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SUMMARY

In higher plants, the process of photosynthesis is localised in the chloroplasts. The membranes, in which the activities are concentrated (the thylakoids) consist, with respect to the lipid part, for over 80% of galactolipids (for structural formulas see p 14). These galactolipids, however, are not synthesised within the thylakoid membranes but in the envelope, the outer membrane structure of the chlorplast. As was already known from previous investigations, the last step in the biosynthesis of galactolipids is the attachment of a galactosyl-moiety from UDPGal into a diacylglycerol molecule. In chapter 3 we tried to elucidate whether this reaction was localised in the outer or in the inner membrane of the chloroplast envelope. Unfortunately, this attempt proved to be unsuccesfull.
In chapter 4 the researches are desribed into the formation of MGDG. The current hypothesis was the formation of MGDG from UDPGal and diacylglycerol according to:

UDPGal + diacylglycerol → MGDG + UDP I

This hypothesis could be confirmed by NMR techniques and by enzyme kinetics. The mechanism of the reaction was investigated and the results are most easily interpreted by introduction of a double displacement or ping-pong reaction. In this reaction UDPGal is the leading substrate and diacylglycerol the second substrate.
A completely new hypothesis was developed with respect to the formation of DGDG. The current hypothesis suggested that DGDG was formed from MGDG and UDPGal by analogy to I. However, many experiments both *in vivo* and with isolated envelopes could not be explained by this hypothesis. In chapter 5 the experiments

are described leading to a new hypothesis for the formation of DGDG as shown in VI:

MGDG + MGDG → DGDG + diacylglycerol VI

Many observations, also from other investigators, could be explained much better by this new hypothesis.

In chapter 6 the lipid composition of envelopes is described and the influence on this composition of the interlipid galactosyltransferase, postulated in VI. By isolation of envelopes in such a way that the interlipid galactosyltransferase was almost completely inhibited, evidence was obtained that the differences in lipid composition may be induced during the isolation procedure. Moreover, the differences in lipid composition between envelopes isolated at pH 7.2 and pH 8.5 are compatible with the results expected from the action of the interlipid galactosyltransferase.

The present data are not sufficient for a full explanation of galactolipid formation *in vivo*. Nevertheless, the differences in enzymic mechanism for the synthesis of MGDG and DGDG, demonstrated in this thesis, and the differences in their dependence on important endogenous and environmental factors may contribute substantially to a further understanding of the ways in which the composition of the major thylakoid lipids is regulated.

SAMENVATTING

In hogere planten vindt de fotosynthese plaats in chloroplasten. De membranen waarin deze aktiviteiten geconcentreerd zijn (de thylakoiden) bestaan voor wat hun lipiden betreft voor ongeveer 80% uit galactolipiden (voor structuurformules zie blz 14). Deze galactolipiden worden echter niet in de thylakoidmembranen gesynthetiseerd, maar in de envelop, het omhullende buitenmembraan systeem van de chloroplast. Uit ouder onderzoek was al bekend dat de laatste stap in de synthese van galactolipiden de koppeling is van een galactosylgroep -afkomstig van UDPGal- aan een diacylglycerol molecuul. In hoofdstuk 3 werd getracht op te helderen of deze koppeling plaatsvindt in de binnenste dan wel in de buitenste envelopmembraan. Hierop kon helaas geen eenduidig antwoord worden verkregen.
In hoofdstuk 4 wordt het onderzoek beschreven naar de vorming van MGDG. De gangbare hypothese was de vorming van MGDG uit diacylglycerol en UDPGal volgens:

UDPGal + diacylglycerol → MGDG + UDP I

Met behulp van NMR techniek en enzymkinetiek kon de bestaande hypothese bevestigd worden. Het reactiemechanisme werd onderzocht en de resultaten maken het waarschijnlijk dat dit geschiedt via een double displacement of ping-pong reactie. Hierin is UDPGal het eerste (leading) en diacylglycerol het tweede substraat.
Een geheel nieuwe hypothese werd ontwikkeld voor de vorming van DGDG. De bestaande hypothese volgens welke UDPGal aan MGDG een tweede galactose molecuul aanbiedt bleek een groot aantal experimenten zowel *in vivo* als met geisoleerde enveloppen niet te kunnen verklaren. In hoofdstuk 5 worden de experimenten beschreven die tot een nieuwe hypothese leidden, betreffende de vorming van

DGDG zoals schematisch weergegeven in VI:

MGDG + MGDG → DGDG + diacylglycerol VI

Het blijkt dat met behulp van deze nieuwe hypothese vele waarnemingen, ook van andere onderzoekers beter verklaard kunnen worden.

In hoofdstuk 6 wordt beschreven hoe de samenstelling is van de geisoleerde enveloppen en wat de invloed hierop zou kunnen zijn van het interlipide galactosyltransferase gepostuleerd in VI. Via isolatie van enveloppen op een zodanige manier dat het interlipide galactosyltransferase vrijwel volledig geremd blijkt te zijn, werden aanwijzingen verkregen dat geisoleerde enveloppen inderdaad een andere lipide samenstelling hebben dan enveloppen waarin het enzym niet actief is. Deze verschillen bleken overeen te komen met de veranderingen die het interlipide galactosyltransferase naar verwachting zou veroorzaken.

De biosynthese van galactolipiden en met name de verschillen in vetzuur samenstelling tussen MGDG en DGDG kunnen niet volledig verklaard worden met de gegevens uit dit proefschrift. Desalniettemin kan het verschil in mechanisme van de MGDG en DGDG synthese, evenals de verschillende afhankelijkheid van de syntheses van belangrijke inwendige en externe factoren, in belangrijke mate bijdragen tot een diepgaander begrip van de manier waarop de samenstelling van de belangrijkste lipiden in de thylakoidmembranen gereguleerd wordt.

Curriculum vitae

Schrijver dezes werd geboren in Eindhoven op 25 december 1947. Na het behalen van het gymnasium β diploma aan het Gymnasium Augustinianum aldaar in 1966, ging hij in datzelfde jaar in Utrecht scheikunde studeren. Het kandidaats (richting S) werd in december 1971 behaald. Het doktoraal programma omvatte het hoofdvak biochemie (isolatie en karakterisering van membraanfrakties van *Entamoeba Invadens* onder leiding van prof dr L. van Deenen) en de bijvakken klinische chemie (onder leiding van prof dr J. Soons) en didaktiek van de scheikunde (onder leiding van dr J. Schuijl). De doktoraalbul werd behaald 1 juli 1974 en per 1 januari 1975 volgde een tijdelijke aanstelling als wetenschappelijk medewerker aan de Katholieke Universiteit Nijmegen.

STELLINGEN

1

Vanwege de geringere geleidbaarheid voor zowel protonen als elektronen voldoet in de thylakoidmembranen een matrix van galactolipiden beter dan een van fosfolipiden.

K. Kano and J.H. Fendler (1978) Biochim. Biophys. Acta 509, 289-299.

2

Voor de veiligheid van bijvoorbeeld een isotopenlaboratorium is het beter globale richtlijnen strikt te handhaven, dan uitgewerkte richtlijnen slechts globaal te handhaven.

3

Nakatani en medewerkers beweren ten onrechte, dat galactolipiden in de thylakoidmembranen niet van buiten af 'zichtbaar' zijn.

H. Nakatani, J. Barber and J.A. Forrester (1978) Biochim. Biophys. Acta 504, 215-225.

4

Door het toenemend gebruik van lithium als therapeutisch middel verdient het aanbeveling dit element als interne standaard in de vlamfotometrische bepaling van natrium en kalium te vervangen door cesium of rubidium.

5

Vanwege de hiaten in onze kennis van de cyclische fotofosforylering en vanwege de geringe bijdrage van dit systeem aan de energiebehoefte van fotoautotrofe organismen verdient het aanbeveling de schema's hiervan niet in de schoolboeken te vermelden.

P.C. Maxwell and J. Biggins (1976) Biochemistry 15, 3975-3981.

6

In tegenstelling tot wat reclameboodschappen suggereren, leidt het nuttigen van extra linolzuur niet tot een gunstigere prognose met betrekking tot hart en vaatziekten.

J. Sheperd, C.J. Packard, J.R. Patsch, A.M. Gotto and D.D. Taunton (1978) J. Clin. Invest. 61, 1582.

7

Het grote verschil in lipide samenstelling dat Hashimoto en Murakami rapporteren in duplo's van thylakoidmateriaal, doet ernstig afbreuk aan de waarde van hun lipide analyse van enveloppen.

H. Hashimoto and S. Murakami (1975) Plant Cell Physiol. 25, 393-421.

8

Uit het feit dat toevoeging van 1 mM cysteïne geen invloed heeft op de verhouding MGDC/DGDG na 1 uur incuberen van Euglena chloroplasten met UDPGal, concluderen Blee en Schanz ten onrechte, dat geen galactolipase activiteit aanwezig is in hun preparaat.

E. Blee and R. Schanz (1978) Plant Science Letters 13, 247-256.

9

De conclusie van Mizuno en Sunderalingam dat het mRNA-tRNA complex evenzeer gestabiliseerd wordt door vorming van een 'wobble' GU basepaar als door vorming van een GC basepaar aan de 5' kant van het anticodon, volgt niet uit hun gegevens.

H. Mizuno and M. Sunderalingam (1978) Nucleic Acids Research 5, 4451-4461.

10

Stelling (eenvoudig te verdedigen); wit: Kd6, pion e5, zwart: Kf5, pion d7.

M. Euwe, Handboek voor de gevorderde schaker I, zesde druk 1962 p 235 Van Stockum, Den Haag.

In hun experimenten met betrekking tot de synthese van galactolipiden vanuit radioactief glycerolfosfaat negeren Joyard en Douce ten onrechte de hoeveelheid MGDG die gevormd wordt in afwezigheid van UDPGal.

J. Joyard and R. Douce (1977) Biochim. Biophys. Acta. 486, 273-285.

Dit proefschrift.

Nijmegen, 27 april 1979.